ME-TOO VALIDATION REPORT

Validation Study for *in vitro* skin corrosion test method using reconstructed human epidermal tissue LabCyte EPI-MODEL24

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LIST OF ACRONYMS AND ABBREVIATIONS

AMED	Agency for Medical Research and Development
BLR	Between-laboratory reproducibility
CAS	Chemical Abstracts Service
CV	Coefficient of variation
D-PBS	Dulbecco's phosphate buffered saline
FBS	Fetal bovine serum
GHS	Global Harmonization System
GLP	Good laboratory practice
JaCVAM	Japanese Centre for the Validation of Alternative Methods
MTT	3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NC	Non-corrosive
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
QC	Quality control
RH	Relative humidity
RHE	Reconstituted human epidermis
RT	Room temperature
SCT	Skin corrosion test
SD	Standard deviation
SLS	Sodium lauryl sulfate
SOP	Standard operating procedure
TER	Transcutaneous electrical resistance
TG	Test guideline
UN	United Nations
VMT	Validation management team
VRM	Validated Reference Method
WLR	Within-laboratory reproducibility

APPENDICES

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1. GOAL STATEMENT

1.1. THE ULTIMATE GOAL

The ultimate goal of this test method is the partial replacement of the acute dermal irritation/corrosion test (1) that enables the identification of corrosive and non-corrosive substances, as well as further categorization of non-corrosive substances into sub-category 1A, according to the UN GHS, as well as a combination of subcategories 1B and 1C.

1.2. PRIMARY GOAL

The primary goal of this me-too validation study is to assess the within- and between-laboratory reproducibility, as well as the predictive capacity, of the LabCyte EPI-MODEL24 skin corrosion test in accordance with the performance standards for the OECD TG 431 (2).

2. OBJECTIVE

OECD TG 431 *in vitro* skin corrosion test guideline was adopted in 2004 to assess the production of irreversible damage to the epidermis following the application of a test chemical (3). Four validated test methods, for which pre-validation, optimization, and validation studies have been completed, are included in this TG: EpiSkin™ Standard Model (SM), EpiDerm™ Skin Corrosivity Test (SCT) (EPI-200), SkinEthic RHE™, and EpiCS®. From these, EpiSkin™ SM and EpiDerm™ SCT (EPI-200) are referred to as validated reference methods (VRM). All four methods use reconstructed human epidermis (RhE) models, and can be used to identify non-corrosive and corrosive substances, and allows partial sub-categorization of corrosives into sub-category 1A and a combination of sub-categories 1B and 1C.

The LabCyte EPI-MODEL24 SCT is another *in vitro* test method that employs a RhE model. The objective of this me-too validation study is to confirm that LabCyte EPI-MODEL24 SCT conforms to the OECD TG 431 by assessing within- and between- laboratory reproducibility, as well as its predictive capacity. The study was performed using the 30 test chemicals listed in the performance standards for the OECD TG 431 as reference chemicals (2).

This validation study was conducted in accordance with the principles and criteria documented in the OECD Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (4), and according to the modular approach to validation (5).

3. BACKGROUND

To date, a number of *in vitro* test methods have been developed as an alternative to the Draize rabbit test (6). In particular, the following tests have been adopted as OECD TGs to predict skin corrosion: OECD TG 430 *in vitro* skin corrosion: transcutaneous electrical resistance (TER) (7), OECD TG 431 *in vitro* skin corrosion: reconstructed human epidermis (RHE) test method (3), and OECD TG 435 *in vitro* membrane barrier test method (8).

As the use of a battery of *in vitro* tests is considered a viable means of improving overall prediction accuracy, there is a clear need for the development of additional *in vitro* test methods. LabCyte EPI-MODEL24 is a RhE tissue model produced using normal human keratinocytes, with a histological structure that is similar to that of the human skin epidermis. The skin irritation test method using LabCyte EPI-MODEL24 has already been included in the OECD TG 439 *in vitro* skin irritation: reconstructed human epidermis test method (9), and the LabCyte EPI-MODEL24 has shown promise as an alternative to animal testing in assessing skin corrosion (10). Therefore, a me-too validation study of the LabCyte EPI-MODEL SCT was conducted in accordance with the performance standards for the OECD TG 431 (2).

4. RECONSTRUCTED HUMAN EPIDERMIS MODEL

4.1. LABCYTE EPI-MODEL24

LabCyte EPI-MODEL24 is a commercially available RhE model produced by Japan Tissue Engineering Co., Ltd. It is comprised of normal human skin-derived keratinocytes cultured on a feeder layer of 3T3-J2 cells that supports proliferation and maintenance of epithelial cells (11). Reconstruction of human cultured epithelial tissue is achieved by cultivating the keratinocytes on an inert filter substrate with a surface area of 0.3 cm^2 at the air-liquid interface for 13 days using an optimized medium containing 5% fetal bovine serum (FBS). This results in the formation of a multilayered structure comprised of fully differentiated stratified cells supported by a layer of proliferating basal cells that mimics that of normal skin. Prior to shipping, LabCyte EPI-MODEL24 tissues are embedded in agarose gel containing nutrient solution. Although raw material lots change over time, quality and performance of each LabCyte EPI-MODEL24 batch released is controlled to ensure highly stable production.

4.2. MODEL SUPPLIER

According to the OECD Good Laboratory Practice (GLP) Consensus Document No. 5 “Compliance of Laboratory Suppliers with GLP Principles”(12), responsibility for the quality and fitness for use of equipment and materials rests entirely with the management of the test facility.

The acceptability of equipment and materials in laboratories complying with GLP must therefore be guaranteed to any regulatory authority to which studies are submitted. In some countries where GLP has been implemented, suppliers belong to national regulatory or

voluntary accreditation schemes that can provide users with additional documentation proving that they are using a test system of defined quality.

5. COMPARISON BETWEEN ESSENTIAL TEST METHOD COMPONENTS OF LABCYTE 24 SCT AND OECD TG 431 VRMS

5.1. COMPARISON BETWEEN FUNCTIONAL CONDITIONS OF LABCYTE EPI-MODEL24 SCT AND VRMS

The OECD document Series on Testing & Assessment No. 219 includes the performance standards for the assessment of proposed similar or modified *in vitro* reconstructed human epidermis (RhE) test methods for skin corrosion testing as described in TG 431 (2). The performance standards consist of: (i) essential test methods components; (ii) minimum list of reference chemicals, and; (iii) defined reliability and accuracy values that the proposed test method should meet or exceed.

The essential test method components section of the performance standards describes the general and functional conditions of the RhE model, and specifically explains the procedural conditions of the VRMs. LabCyte EPI-MODEL24 meets these general and functional conditions, as they are the same as those described in the performance standards for the OECD TG 439 skin irritation test (13), to which LabCyte EPI-MODEL24 has been included. In short, LabCyte EPI-MODEL24 is produced with non-transformed human keratinocytes seeded in culture inserts with a porous synthetic membrane. The reconstructed epithelium consists of multiple layers of viable epithelial cells (*stratum basale*, *stratum spinosum*, *stratum granulosum*) organized under a functional *stratum corneum* (Figure 1). These cells mimic the histological, morphological, biochemical and physiological properties of the epidermis, as observed by immunostaining against differentiation markers (filaggrin, loricrin), adhesion molecules (claudin-1, e-cadherin), and cytokeratins (keratin 5 and keratin 10) (Figure 2). LabCyte EPI-MODEL24 is cultured at the air-liquid interface, allowing topical application of test

chemicals to a tissue surface that is in direct contact with air, similarly to *in vivo* conditions. The *stratum corneum* is multilayered and contains the essential lipid profile that produces a functional barrier resistant to sodium dodecyl sulphate (SDS) penetration, as estimated by IC_{50} (Table 1).

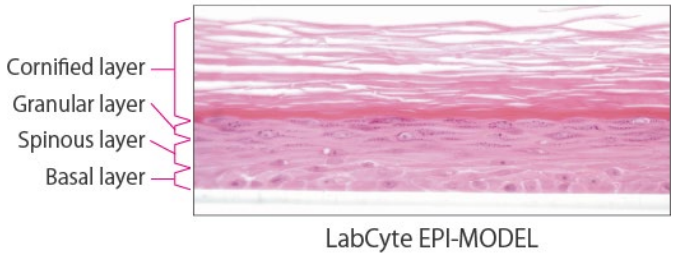

Figure 1 - Histological structure of LabCyte EPI-MODEL24

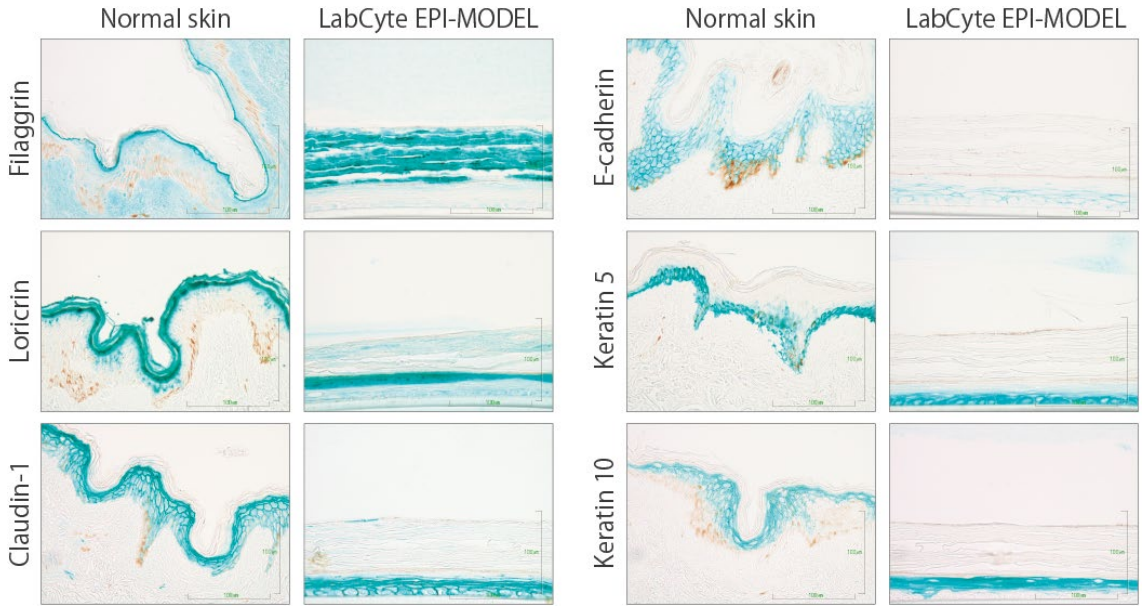

Figure 2 - Immunostaining of LabCyte EPI-MODEL24

Table 1 - Comparison of QC batch release criteria

	Lower acceptance limit	Upper acceptance limit
EpiSkin™ (SM) (18 hours treatment with SDS)	IC_{50} = 1.0 mg/mL	IC_{50} = 3.0 mg/mL
EpiDerm™ SCT (EPI-200) (1% Triton X-100)	ET_{50} = 4.0 hours	ET_{50} = 8.7 hours
LabCyte EPI-MODEL24 SCT (18 hours treatment with SDS)	IC_{50} = 1.4 mg/mL	IC_{50} = 4.0mg/mL

MTT assay is used to quantify tissue viability. Viable cells of the RhE reduce the vital dye MTT into a blue MTT formazan precipitate that can be extracted from the tissue using isopropanol, and quantified using standard optical density (OD) measurement. An acceptability range (upper and lower limit, Table 2) for the negative control OD values was established according to the performance standards requirements.

Table 2 - Comparison of acceptability ranges for negative control OD values

	Lower acceptance limit	Upper acceptance limit
EpiSkin™ (SM)	≥ 0.6	≤ 1.5
EpiDerm™ SCT (EPI-200)	≥ 0.8	≤ 2.8
LabCyte EPI-MODEL24 SCT	≥ 0.7	≤ 2.5

5.2. COMPARISON BETWEEN PROCEDURAL CONDITIONS OF LABCYTE EPI-MODEL24 SCT AND VRMS

A comparison between test components of the LabCyte EPI-MODEL24 SCT and the two OECD TG431 VRMs are shown in Table 3.

Table 3 - Description of RHE SCT test components

Test component	LabCyte EPI-MODEL24 SCT	EpiSkin™	EpiDerm™ SCT
Pre-exposure	Overnight incubation at 37°C, 5% CO_2, 95% RH	3-24 hours incubation at 37° C	Optional overnight incubation at 37°C, 5% CO_2, 95% RH
Number of tissue replicates	2 per exposure time	At least 2 per exposure time	2-3 per exposure time
Treatment dose and application	Liquids: 50 µL (167 µL/cm²) Solids: 50 µL of H_2O + 50 mg ± 2 mg (167 mg/cm²)	Liquids and viscous: 50 µL ± 3 µL (131.6 µL/cm²) Solids: 20 ± 2 mg (52.6 mg/cm²) + 100 µL ± 5 µL NaCl solution (9 g/L) Waxy/sticky: 50 ± 2 mg (131.6 mg/cm²) with a nylon mesh	Liquids: 50 µL (79.4 µL/cm²) with or without a nylon mesh Semisolids: 50 µL (79.4 µL/cm²) Solids: 25 µL H_2O (or more if necessary) + 25 mg (39.7 mg/cm²) Waxes: flat "disk like" piece of ca. 8 mm diameter placed atop the tissue wetted with 15 µL H_2O.
Pre-check for color interference	50 µL (liquid) or 50 mg (solid) + 500 µL H_2O mixed for 60 min at 37°C, 5% CO_2, 95% RH → if solution becomes colored, living adapted controls should be performed	10 µL (liquid) or 10 mg (solid) + 90 µL H_2O mixed for 15 min at RT → if solution becomes colored, living adapted controls should be performed	50 µL (liquid) or 25 mg (solid) + 300 µL H_2O for 60 min at 37°C, 5% CO_2, 95% RH → if solution becomes colored, living adapted controls should be performed

Test component	LabCyte EPI-MODEL24 SCT	EpiSkin™	EpiDerm™ SCT
Pre-check for direct MTT reduction	50 µL (liquid) or 50 mg (solid) + 500 µL MTT 0.5 mg/mL for 60 min at 37°C, 5% CO_2, 95% RH → if solution turns blue/purple, freeze-killed adapted controls should be performed	50 µL (liquid) or 20 mg (solid) + 2 mL MTT 0.3 mg/mL solution for 180 ± 5 min at 37°C, 5% CO_2, 95% RH → if solution turns blue/purple, water-killed adapted controls should be performed	50 µL (liquid) or 25 mg (solid) + 1 mL MTT 1 mg/mL solution for 60 min at 37°C, 5% CO_2, 95% RH → if solution turns blue/purple, freeze-killed adapted controls should be performed
Test chemical exposure time	3 min at RT, and 60 min at 37°C, 5% CO_2, 95% RH	3 min, 60 min (± 5 min) and 240 min (± 10 min) In ventilated cabinet Room temperature (RT, 18-28°C)	3 min at RT, and 60 min at 37°C, 5% CO_2, 95% RH
Negative control	50 µL H_2O Tested with every exposure time	20 µL NaCl solution (9 g/L) Tested with every exposure time	50 µL H_2O Tested with every exposure time
Positive control	50 µL 8N KOH Tested with every exposure time	50 µL Glacial acetic acid Tested only for 4 hours	50 µL 8N KOH Tested with every exposure time
MTT assay	500 µL 0.5 mg/mL	2 mL 0.3 mg/mL	300 µL 1mg/ml
Cell viability threshold	15% and 50% at 3 min 15% at 60 min	35% at 3, 60, 240 min	25% and 50% at 3 min 15% at 60 min
Detection and correction of MTT interference	Colored: using viable tissue MTT reducer: using killed tissue	Colored: using viable tissue MTT reducer: using killed tissue	Colored: using viable tissue MTT reducer: using killed tissue

Test component	LabCyte EPI-MODEL24 SCT	EpiSkin™	EpiDerm™ SCT
Acceptance criteria (SD)	1. Mean OD of the tissue replicates treated with the negative control (H_2O) should be ≥ 0.7 and ≤ 2.5 for every exposure time. 2. Mean viability of the tissue replicates exposed for 1 hour with the positive control (8N KOH) , expressed as % of the negative control should be ≤ 15%. 3. In the range 20-100% viability and ODs ≥ 0.3, the difference of viability between the two tissue replicates should not exceed 30%	1. Mean OD of the tissue replicates treated with the negative control (NaCl) should be ≥ 0.6 and ≤ 1.5 for every exposure time. 2. Mean viability of the tissue replicates exposed for 4 hours with the positive control (glacial acetic acid) , expressed as % of the negative control should be ≤ 20%. 3. In the range 20-100% viability and for ODs ≥ 0.3, difference of viability between the two tissue replicates should not exceed 30%	1. Mean OD of the tissue replicates treated with the negative control (H_2O) should be ≥ 0.8 and ≤ 2.8 for every exposure time. 2. Mean viability of the tissue replicates exposed for 1 hour with the positive control (8N KOH) , expressed as % of the negative control should be ≤ 15%. 3. In the range 20-100% viability, the coefficient of variation (CV) between tissue replicates should be ≤ 30%

5.2.1. Application of test chemical and control substances

According to the performance standards for the OECD TG 431 (2), at least two tissue replicates should be used for each test chemical and each control substance, for each exposure time in each run. Moreover, a minimum of 50 μL or 50 mg of test chemical should be applied to ensure that it uniformly covers the surface of the epidermis. LabCyte EPI-MODEL24 SCT meets these requirements, as tests are conducted using two tissue replicates, and 167 μL/cm^2 or 167 mg/cm^2 of liquid and solid chemicals, respectively, are applied to the tissue.

5.2.2. Cell viability measurements

According to the performance standards for the TG 431, the MTT assay should be used to

measure tissue viability as an endpoint for the prediction of skin corrosion (2). MTT is a tetrazolium dye that can be reduced by cellular dehydrogenase to produce insoluble MTT formazan as a blue precipitate that can be extracted from the tissue using a solvent. Since cellular dehydrogenase is rapidly inactivated by damaged cells, the degree of coloring by formazan dye directly correlates to cell viability, which consequently, can be quantified by measuring the absorbance of the colored solvent solution.

Test substances may interfere with the MTT assay by coloring the tissues in the same absorbance OD range as formazan (570 ± 30 nm), or by directly reducing the formazan dye (MTT reducers). Because this interference can lead to a false estimate of tissue viability, pre-checks should be performed before testing to allow for identification of color interfering chemicals and MTT reducers. In the LabCyte EPI-MODEL24 SCT, color interfering chemicals are identified by mixing the test substance with water, and further performing tests using viable tissues control in case the water becomes colored. MTT reducers, on the other hand, are identified by mixing the test substance with MTT solution, and further performing tests using freeze-killed tissues in case the MTT solution is reduced and forms a blue precipitate.

The VMT determined that the MTT assay protocol for the LabCyte EPI-MODEL24 SCT was similar to those of OECD TG 431 VRMs, and was in concordance with the performance standards requirements for the OECD TG 431.

5.2.3. Acceptability criteria

According to the performance standards (2), the OD and cell viability of negative and positive controls, respectively, should fall within a determined range established from historical values. Likewise, the variability between tissue replicates must also fall within accepted limits. Should any of the values above fall outside the accepted ranges, the test run is

considered non-qualified and should be repeated.

The acceptability criteria for LabCyte EPI-MODEL24 SCT were determined prior to the validation study, and were based on the results of a preliminary study (10), therefore complying with the requirements of the performance standards.

5.2.4. Interpretation of results and prediction model

The LabCyte EPI-MODEL24 SCT adopts the prediction model of EpiDerm™ SCT, one of the OECD TG 431 VRMs, as it proved to be suitable for the corrosion prediction of chemicals in a preliminary study (10), (Annex 1).

5.3. SIMILARITY BETWEEN LABCYTE EPI-MODEL24 AND OECD TG 431 VRMS

Based on the points described in sections 5.1 and 5.2, the VMT considers the LabCyte EPI-MODEL24 SCT to be a derivative of the VRMs adopted by the OECD TG 431.

6. VALIDATION MANAGEMENT STRUCTURE

The validation study was, in part, funded by the Agency for Medical Research and Development (AMED). The management structure is shown in Figure 3 (Appendix 1).

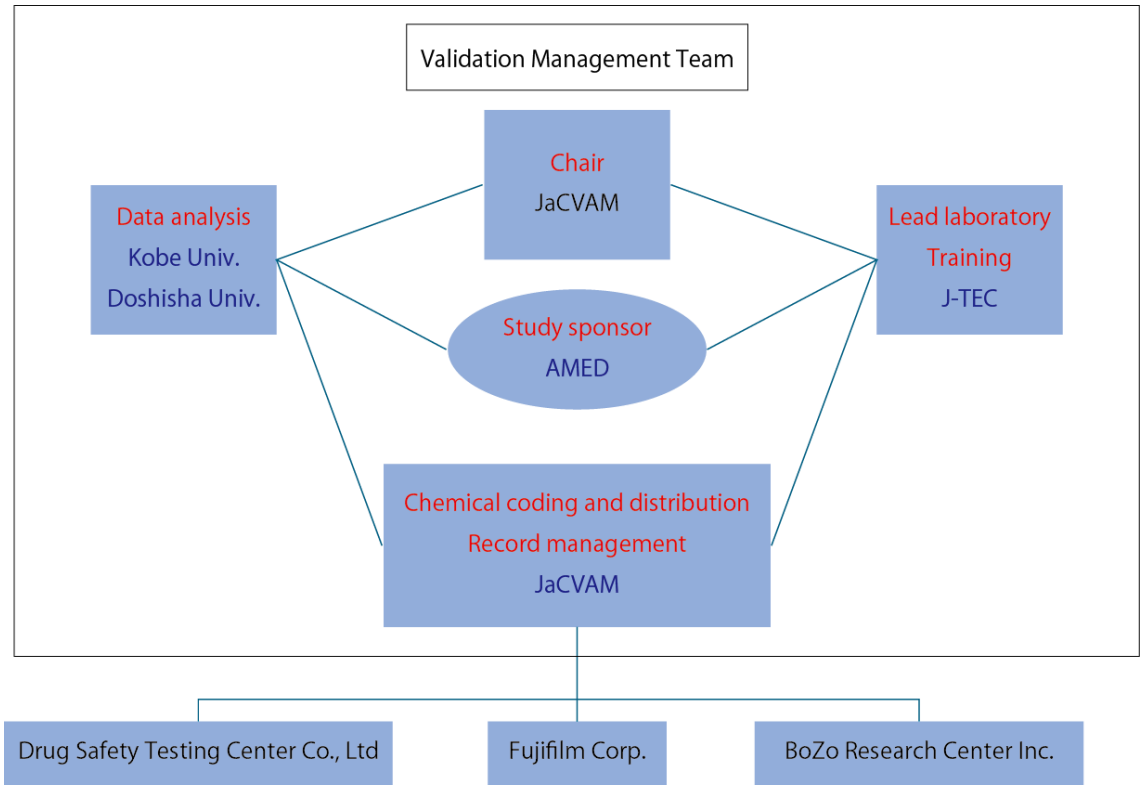

Figure 3 - Management structure for the LabCyte EPI-MODEL24 SCT validation study

6.1. VALIDATION TEAM MANAGEMENT

A validation management team (VMT) was established to make this study scientifically pertinent, and to ensure its smooth progress.

The VMT is led by a trial coordinator, and consists of a chemical management, a data analysis group, a record management, and the lead laboratory (assay developer). The lead laboratory provides support for the participating laboratories. The VMT was responsible for preparing,

reviewing, and finalizing the study plan and the study protocol. In addition, the VMT was also responsible for managing the validation study by monitoring its progress, assuring the quality of study records, and communicating with the participating laboratories.

The VMT played a central role in overseeing the validation study, including the planning and implementation of the following:

1. Goal statement
2. Project plan, including objectives
3. Study protocol and amendments
4. Outcome of quality control audits
5. Test chemicals
6. Data management procedures
7. Timeline and study progression
8. Data collection and analysis
9. Study interpretation and conclusions
10. Reports and publications

6.2. TRIAL COORDINATOR

The assigned trial coordinator Hajime Kojima (Japanese Center for the Validation of Alternative Test Method, JaCVAM) was responsible for the operational management of this validation study. That included the preparation of the study plan and study protocol, the compilation of the test chemical list, and convening ad hoc VMT meetings.

6.3. CHEMICAL SELECTION, ACQUISITION, CODING AND DISTRIBUTION

The chemical management was led by Hajime Kojima, and included other JaCVAM staff members. The validation study was conducted in accordance with the performance standards

for the skin corrosion testing as described in TG 431, therefore, the chemical management group was not required to select the test chemicals for this study. The test chemicals were encoded and distributed to the participating laboratories by JaCVAM. Thus, participating laboratories performed the tests without knowing the identity of the chemicals, nor how they were coded.

6.4. DATA ANALYSIS GROUP

The data analysis group was led by Takashi Omori (Kobe University), and included other members from Kobe University and Doshisha University. All members declared no conflict of interest associated with this validation study. The data analysis group was responsible for the statistical processing and data analysis, from a third-party stand point. The group also ensured that measured values were appropriately recorded in the data sheets (Appendix 2).

6.5. RECORD MANAGEMENT

The record management was led by Hajime Kojima, and included other JaCVAM staff members. The record management prepared and distributed protocols, test substance preparation forms and blank data sheets, among other documents, to all participating laboratories. They also collected filled out forms and data sheets after the test completion, pointing out omissions or flaws in the recordings, if any, and requested correction of such errors.

6.6. LEAD LABORATORY

As the lead laboratory, Japan Tissue Engineering Co., Ltd (J-TEC) provided support to the participating laboratories.

6.7. PARTICIPATING LABORATORIES

The three laboratories listed below participated in this validation study by performing the LabCyte EPI-MODEL24 SCT using the 30 test chemicals listed in the performance standards for the OECD TG 431 (2).

Laboratory A (Lab A): Drug Safety Testing Center Co. Ltd (Saitama)

Laboratory B (Lab B): Fujifilm Corp. (Kanagawa)

Laboratory C (Lab C): Bozo Research Center Inc. (Tokyo)

All three laboratories are located in Japan, 230-330 km away from J-TEC's tissue manufacturing facility. All tissues models were delivered to the testing laboratories by truck.

Participating laboratories underwent training for the LabCyte EPI-MODEL24 SCT, and receive participation approval by the VMT prior to the start of this validation study. J-TEC, the lead laboratory, is a subsidiary of Fujifilm Corp. Fujifilm Corp. participated as an independent laboratory and declared no conflict of interest associated with this validation study, as all its operations, including testing facilities, are not located in the same premises as J-TEC's.

6.8. SPONSORSHIP

The study was managed and funded jointly by the Agency for Medical Research and Development (AMED), JaCVAM, J-TEC and the participating laboratories.

1. AMED provided funding.
2. JaCVAM provided funding for:
   - Management of the validation study.
   - Purchase, coding and distribution of chemicals.
   - Independent data quality control audit.

- Publication of the validation study results.

3. J-TEC provided funding for:

- Training participating laboratories.
- Independent LabCyte EPI-MODEL quality control audits.
- Funding the participating laboratories.

7. STUDY DESIGN

7.1. TEST CHEMICALS

7.1.1. Chemical selection

The 30 reference chemicals listed in the performance standards (2) were used to determine whether the reproducibility and predictive capacity of LabCye EPI-MODEL24 SCT are equal to or better than the defined minimum values for the TG 431 VRMs. Seven of these chemicals (underlined in Table 4) were selected for a training phase that took place prior to the validation study.

7.1.2. Coding and distribution

The thirty reference chemicals were tested in three independent runs, at three independent laboratories. To ensure a blind evaluation was performed, the 30 chemicals were coded in a total of 270 test substances (Table 4, Appendix 3) that were distributed to the chemical master at the participant laboratories in three separate sets of 30 substances each by JaCVAM. All essential information about the test chemicals were provided including physical state, weight or volume of sample and storage instructions. The chemical master was responsible for storing each chemical in accordance with the storage instructions and received sealed safety information, including Material Safety Data Sheets (MSDS; Appendix 4), which specified hazard identification, exposure control, and personal protection for each chemical. The test chemicals were delivered directly to the chemical master, who was not shown any MSDS. The study director was to refer to MSDS only in the event of an accident. The laboratory technicians who actually performed the testing were not to be

informed, even if the study director referred to the MSDS.

7.2. DEFINED RELIABILITY AND ACCURACY VALUES

All thirty reference chemicals listed in Table 4 were tested at each of the three participating laboratories, in order to evaluate the reliability and relevance of the LabCyte EPI-MODEL24 SCT. Each laboratory performed three independent runs for each test chemical with different tissue batches and at sufficient time intervals. Each run comprised three concurrently tested replicates for each test chemical, negative control and positive control.

Table 4 - OECD TG 431 reference chemicals list

No.	Chemicals	State	CAS number (Supplier)	Codes		
				Lab A	Lab B	Lab C
	Non-corrosive chemicals based *on vivo* results					
1	Phenylethyl bromide	Liquid	103-63-9 (Sigma-Aldrich)	LSA02 LSA110 LSB240	LSB39 LSB135 LSA229	LSC64 LSC162 LSC285
2	4-Amino-1,2,4-trizole	Solid	584-13-4 (Sigma-Aldrich)	LSA14 LSA104 LSB251	LSB44 LSB141 LSA223	LSC65 LSC163 LSC287
3	4-(methylthio)-benzaldehyde	Liquid	3446-89-7 (Alfa Aesar)	LSA23 LSA123 LSB252	LSB45 LSB142 LSA202	LSC78 LSC187 LSC289
4	Lauric acid	Solid	143-07-7 (Sigma-Aldrich)	LSA24 LSA116 LSB253	LSB54 LSB159 LSA214	LSC61 LSC164 LSC272
5	1,9-Decadiene	Liquid	1647-16-1 (Sigma-Aldrich)	LSA29 LSA118 LSB255	LSB55 LSB160 LSA224	LSC83 LSC178 LSC273
6	2,4-Dimethylaniline	Liquid	95-68-1 (Sigma-Aldrich)	LSA07 LSA107 LSB239	LSB40 LSB145 LSA208	LSC72 LSC183 LSC268

No.	Chemicals	State	CAS number (Supplier)	Codes		
				Lab A	Lab B	Lab C
7	3,3-Dithiopropionic acid	Solid	1119-62-6 (Sigma-Aldrich)	LSA08 LSA111 LSB244	LSB51 LSB146 LSA225	LSC73 LSC174 LSC269
8	Methyl palmitate	Solid	112-39-0 (Sigma-Aldrich)	LSA25 LSA112 LSB245	LSB52 LSB131 LSA230	LSC87 LSC175 LSC270
9	2-Hydroxyiso-butyric acid	Solid	594-61-6 (Sigma-Aldrich)	LSA28 LSA113 LSB254	LSB53 LSB132 LSA207	LSC85 LSC176 LSC271
10	Sodium undecylenate (33%)	Liquid	3398-33-2 (Santa Cruz)	LSA30 LSA130 LSB231	LSB31 LSB149 LSA228	LSC89 LSC177 LSC276
	Combination of UN GHS Sub-categories 1B and 1C based on *in vivo* results					
11	Glyoxylic acid monohydrate	Liquid	563-96-2 (Sigma-Aldrich)	LSA01 LSA101 LSB232	LSB41 LSB150 LSA201	LSC90 LSC168 LSC262
12	Lactic acid	Solid	598-82-3 (Sigma-Aldrich)	LSA11 LSA102 LSB237	LSB42 LSB133 LSA204	LSC84 LSC169 LSC263
13	Sodium bisulphate monohydrate	Liquid	10034-88-5 (Sigma-Aldrich)	LSA12 LSA103 LSB238	LSB59 LSB134 LSA211	LSC79 LSC190 LSC274
14	Ethanolamine	Viscous	141-43-5 (Sigma-Aldrich)	LSA17 LSA117 LSB256	LSB38 LSB148 LSA217	LSC88 LSC179 LSC267
15	60/40 Octanoic/decanoic acid	Liquid	68937-75-7 (Mixture)	LSA19 LSA129 LSB241	LSB35 LSB144 LSA219	LSC77 LSC171 LSC280
	Octanoid acid	Liquid	124-07-2 (Sigma-Aldrich)			
	Decanoid acid	Solid	334-48-5 (Sigma-Aldrich)			
16	Hydrochloric acid (14.4%)	Liquid	7647-01-0 (Sigma-Aldrich)	LSA09 LSA106 LSB242	LSB37 LSB147 LSA210	LSC86 LSC161 LSC266

No.	Chemicals	State	CAS number (Supplier)	Codes		
				Lab A	Lab B	Lab C
17	Fluoroboric acid	Liquid	16872-11-0 (Sigma-Aldrich)	LSA04 LSA105 LSB235	LSB60 LSB143 LSA212	LSC82 LSC171 LSC275
18	Propionic acid	Liquid	79-09-4 (Sigma-Aldrich)	LSA18 LSA108 LSB257	LSB56 LSB151 LSA209	LSC66 LSC172 LSC286
19	2-tert-Butylphenol	Liquid	88-18-6 (Alfa Aesar)	LSA22 LSA109 LSB259	LSB57 LSB152 LSA218	LSC67 LSC173 LSC288
20	Cyclohexyl amine	Liquid	108-91-8 (Sigma-Aldrich)	LSA10 LSA122 LSB260	LSB32 LSB153 LSA222	LSC81 LSC180 LSC281
	UN GHS Sub-category 1A based on *in vivo* results					
21	Acrylic acid	Liquid	79-10-7 (Sigma Aldrich)	LSA15 LSA114 LSB233	LSB34 LSB154 LSA226	LSC74 LSC186 LSC261
22	Bromoacetic acid	Solid	79-08-3 (Fluka)	LSA03 LSA115 LSB234	LSB36 LSB140 LSA216	LSC75 LSC185 LSC264
23	Boron trifluoride dehydrate	Liquid	13319-75-0 (Sigma-Aldrich)	LSA16 LSA119 LSB250	LSB47 LSB138 LSA227	LSC80 LSC189 LSC265
24	Phenol	Solid	108-95-2 (Sigma-Aldrich)	LSA26 LSA120 LSB258	LSB48 LSB139 LSA215	LSC62 LSC167 LSC278
25	Phosphorus tribromide	Liquid	7789-60-8 (Sigma-Aldrich)	LSA27 LSA128 LSB249	LSB33 LSB158 LSA203	LSC63 LSC181 LSC283
26	Silver nitrate	Solid	7761-88-8 (Sigma-Aldrich)	LSA05 LSA121 LSB246	LSB43 LSB136 LSA213	LSC68 LSC165 LSC277
27	Formic acid	Liquid	64-18-6 (Fluka)	LSA06 LSA124 LSB247	LSB46 LSB137 LSA220	LSC69 LSC166 LSC279

No.	Chemicals	State	CAS number (Supplier)	Codes		
				Lab A	Lab B	Lab C
28	Dichloroacetyl chloride	Liquid	79-36-7 (Sigma-Aldrich)	LSA13 LSA125 LSB248	LSB49 LSB155 LSA221	LSC70 LSC182 LSC282
29	Sulphuric acid (98%)	Liquid	7664-93-9 (Merck)	LSA20 LSA126 LSA236	LSB50 LSB156 LSA205	LSC71 LSC184 LSC284
30	N,N-Dimethyl dipropylene triamine	Liquid	10563-29-8 (Sigma-Aldrich)	LSA21 LSA127 LSB243	LSB58 LSB157 LSA206	LSC76 LSC188 LSC290
	Potassium hydroxide (5%)	Liquid	1310-58-3 (Sigma-Aldrich)	Positive control		

Underlined chemicals: substances used in the training phase of this validation study.

7.2.1. Study quality criteria

In the event a test result fails to meet the acceptance criteria for a control or test chemical, or is considered invalid due to any reason, a maximum of two additional retests are permitted to complement the dataset. In other words, since retesting requires concurrent testing of negative and positive controls, a maximum number of two additional runs are permitted for each test chemical.

It is conceivable that, even after retesting, one or more participating laboratories will fail to obtain three valid runs for each test chemical, thus resulting in an incomplete data matrix. A dataset is considered valid, however, as long as the following criteria are met:

1. All relevant reference chemicals (24 for category 1 vs. non-corrosive; 30 for sub-category 1A vs. sub-category 1B-and-1C vs. non-corrosive) should have at least one complete test sequence in one laboratory;

2. Each of at least three participating laboratories should have a minimum of 85% complete test sequences (for 24 reference chemicals: three incomplete test sequences are

allowed per laboratory; 30 reference chemicals: four incomplete test sequences are allowed per laboratory); and

3. At least 90% of all test sequences from at least three laboratories need to be complete (24 reference chemicals tested in three laboratories: a total of seven incomplete test sequences are allowed; for 30 reference chemicals tested in three laboratories: a total of nine incomplete test sequences are allowed).

In this context, a test sequence consists of the total number of independent tests performed for a single reference chemical in a single laboratory, including any retesting (a total of 3 to five tests). A test sequence may include both qualified and non-qualified tests. A complete test sequence consists of a test sequence containing three qualified tests. A test sequence containing less than three qualified tests is considered as incomplete.

7.2.2. Within-laboratory reproducibility

The VMT set the target value for within-laboratory reproducibility according to the requirements of the performance standards. For a test proposed to discriminate sub-category 1A, a combination of sub-categories 1B and 1C, as well as non-corrosive chemicals, the concordance of predictions obtained in different independent tests of the 30 reference chemicals should be equal or higher than 80% (2).

7.2.3. Between-laboratory reproducibility

Likewise, the between-laboratory reproducibility target value was also set according to the requirements of the performance standards. For a test proposed to discriminate sub-category 1A, a combination of sub-categories 1B and 1C, as well as non-corrosive chemicals, the concordance of predictions between a minimum of three laboratories obtained for the 30 reference chemicals should be equal or higher than 70%.

7.2.4. Predictive capacity

The VMT determined that the target values for predictive capacity (sensitivity, specificity, and overall accuracy) should be equal to or better than the values derived from the OECD TG 431 VRMs, and at the same time, based on the performance of LabCyte EPI-MODEL24 SCT historical data (underlined chemicals in Annex 1). The target value for sensitivity, specificity, and overall accuracy along with the criteria for the performance of LabCyte EPI-MODEL24 SCT and other models using the 30 reference chemicals are shown in Table 5.

Table 5 - Required predictive capacity for LabCyte EPI-MODEL24 SCT

	EpiSkin™	**EpiDerm™**	**LabCyte EPI-MODEL24 historical data[1]**	**LabCyte EPI-MODEL24 requirements**
Sensitivity (for predictions C vs. NC)	≥ 95%	≥ 95%	100%	≥ 95%
Correctly classified 1A[2]	≥ 80%	≥ 90%	87%	≥ 80%
1A underclassified 1B-and-1C[2]	≤ 20%	≤ 10%	13%	≤ 20%
1A underclassified NC	0%	0%	0%	0%
Correctly classified 1B-and-1C	≥ 80%	≥ 55%	67%	≥ 55%
1B-and-1C overclassified 1A	≤ 20%	≤ 45%	33%	≤ 45%
1B-and-1C underclassified NC	≤ 5%	≤ 5%	0%	≤ 5%
Specificity	≥ 70%	≥ 70%	87%	≥ 70%
NC overclassified 1A	≤ 5%	≤ 5%	0%	≤ 5%
NC overclassified 1B-and-1C	≤ 30%	≤ 30%	13%	≤ 30%
Accuracy (C vs. NC)	≥ 87%	≥ 87%	96%	≥ 87%
Accuracy (1A vs. 1B-and-1C vs. NC)	≥ 78%	≥ 72%	80%	≥ 72%

[1]Calculated using the 30 reference chemicals included in the lead laboratory's data base (Annex 1).

[2]Based on the updated EpiDerm prediction model within TG 431 (3) published in 2016 and on a publication from Desprez et al. [12], correctly classified 1A would be 83.4% and 1A underclassified 1B-and-1C would be 16.6%.

7.3. DATA COLLECTION, HANDLING, AND ANALYSIS

Working in close collaboration with JaCVAM, the data analysis group collected and organized the data using custom data collection software, which included decoding the test chemicals and performing statistical analyses using tools that were approved by the VMT.

7.4. QUALITY ASSURANCE AND GLP

All participating laboratories conducted the tests in the principle of GLP.

Quality assurance of all data and records was performed by JaCVAM. After completion of all tests, study documents were submitted to the trial coordinator, and only data sheets were forwarded by email to the data analysis group. The trial coordinator reviewed all the study documents and clarified illegible or unclear content, if any, by contacting participant laboratories by email or telephone.

8. PROTOCOL

8.1. PROTOCOL OF THE LABCYTE EPI-MODEL24 SCT

Prediction of skin corrosion potential of test chemicals using the LabCyte EPI-MODEL24 SCT was performed using the standard operating procedure (SOP) version 1.6 (Appendix 5.) . The same SOP was used to estimate the predictive performance of LabCyte EPI-MODEL24 SCT using 79 test chemicals (Annex 1).

The LabCyte EPI-MODEL24 tissues shipped to the participating laboratories were aseptically removed from the agarose medium, placed in wells containing 500 μL of assay medium, and then incubated overnight at 37°C in a humidified atmosphere of 5% CO_2 in air.

8.1.1. Application of liquid and solid chemicals

After incubation, the tissues were topically exposed to either 50 μL of a liquid test chemical, applied with a micropipette, or 50 μL of H_2O and 50 mg of a solid test chemical weighed in advance using a precision balance. Each test chemical was applied to four tissue replicates. Additionally, four tissues were treated with 50 μL of distilled water as negative controls, and two with 8 N KOH as positive controls. Two tissues exposed to the test chemical, and two tissues exposed to the negative control were incubated for three minutes. The remaining test chemical, and negative and positive control replicates (two for each group) were incubated for 60 minutes. Next, each tissue was carefully rinsed at least 10 times with Dulbecco's Phosphate Buffer (D-PBS) applied from a washing bottle to remove any residual test chemical from the tissue surface. The tissues were blotted, placed in new wells containing 500 μL of MTT medium (MTT 0.5 g/mL in assay medium), and incubated for three hours at 37°C in a humidified atmosphere of 5% CO_2 in air.

After incubation in MTT medium, the epidermis tissues were transferred from the culture inserts to 1.5 mL microtubes containing 300 µL of isopropanol. The microtubes were incubated for at least 15 hours in a dark and cold place in order to completely extract the MTT formazan. Finally, 200 µL of the isopropanol extraction solution were placed in a microtiter plate and the optical density (OD) value was measured at 570 nm, and 650 nm as reference absorbance, with propanol as blank. The cell viability was calculated as the ratio of the OD of the test chemical to the OD of the negative control, as in the formula below:

$$\text{Cell viability (\%)} = \frac{Test\ chemical\ OD}{Negative\ control\ OD} \times 100$$

The mean of two replicates was used to predict skin corrosion potential of the test chemical according to the prediction model.

8.1.2. Detecting and correcting chemical interference with MTT endpoints

Two types of test chemicals interfere with the MTT assay and affect the test endpoints:

1. Chemicals that stain epidermal tissues; and
2. Chemicals able to directly reduce MTT

Test chemicals that stain the epidermis tissues could possibly dissolve in isopropanol and affect OD measurements. Test chemicals that directly reduce MTT could possibly affect OD measurements if the test chemical is still present in the epidermis when the MTT assay is performed. A procedure to detect such test chemicals is described below.

8.1.2.1. Detection of chemicals that stain epidermis tissues

<u>Step 1 – Preliminary test</u>

Test chemicals (liquids: 50 µL, solids: 50 mg) were added to 500 µL of distilled water in a 24-well plate. Untreated distilled water was used as control. The 24-well plate was then

incubated for four hours at 37°C in a humidified atmosphere of 5% CO_2 in air. After incubation, the mixture was gently shaken and visually examined for staining of the distilled water. If the color of the solution did not significantly change, the chemical was considered to not have the potential to stain the tissue. However, if the color of the solutions changed, a functional check with viable tissues (step 2) was performed.

Step 2 – Measurement of tissue staining for OD correction

Chemicals that clearly stained the distilled water in step 1 were applied to the surface of the epidermis tissue (liquids: 50 µL, solids: 50 mg). The procedure briefly described in section 8.1.1 was then followed, however, with assay medium as a replacement to MTT medium so epidermis tissue staining could be evaluated. The corrected OD was calculated using the formula below:

$$\textbf{Corrected OD} = \mathbf{A} - (\mathbf{B} - \mathbf{C})$$

Where:

A is the OD of tissue exposed to the test chemical using MTT medium;

B is the mean OD of tissue exposed to the test chemical using assay medium instead of MTT medium; and

C is the mean OD of the tissue exposed to the negative control using assay medium instead of MTT medium.

If the OD of $(B - C)$, or a corrected OD, was below zero, they were considered zero.

8.1.2.2. Detection of chemicals that directly reduce MTT

Step 1 – Preliminary test

Test chemicals (liquids: 50 µL, solids: 50 mg) were added to 500 µL of MTT medium in a 24-well plate. Untreated MTT medium was used as control. The 24-well plate was then

incubated for 60 minutes at 37°C in a humidified atmosphere of 5% CO_2 in air. After incubation, the mixture was gently shaken and visually examined for staining of the MTT medium. If the color of the MTT solution did not significantly change, the chemical was considered to not have the potential to stain the tissue. However, if the color of the MTT solution turned blue/purple, a functional check with freeze-killed tissues (step 2) was performed.

Step 2 – Measurement of direct MTT reduction for OD correction

Chemicals that clearly changed the color of the MTT medium to blue/purple in step 1, were applied to the surface of the epidermis tissues (liquids: 50 µL, solids: 50 mg). The procedure briefly described in section 8.1.1 was then followed, however, with freeze-killed tissue as a replacement to viable tissues. The freeze-killed tissues were prepared by repeating twice a freezing cycle at -80°C or lower for one hour, and 37°C for 30 minutes. The corrected OD was calculated using the formula below:

$$\textbf{Corrected OD} = \mathbf{A} - (\mathbf{B} - \mathbf{C})$$

Where:

A is the OD of viable tissue exposed to the test chemical;

B is the mean OD of freeze-killed tissue exposed to the test chemical; and

C is the mean OD of freeze-killed tissue exposed to the negative control.

If the OD of $(B - C)$, or a corrected OD, was below zero, they were considered zero.

8.2. PREDICTION MODEL FOR THE LABCYTE EPI-MODEL24 SCT

The corrosive potential of test chemicals was predicted from the relative mean tissue viabilities obtained after three minutes, as well as 60 minutes treatment, compared to the negative control tissues concurrently treated with distilled water. The prediction model for the LabCyte EPI-MODEL24 SCT is shown in Table 6.

Table 6 - LabCyte EPI-MODEL24 SCT prediction model

Step 1			Step 2	
Cell viability		Prediction	Cell viability	Sub-category
3 min	60 min		3 min	
< 50%		Corrosive	< 15%	1A
≥ 50%	< 15%	Corrosive	≥ 15%	1B-and-1C
	≥ 15%	Non-corrosive		

8.3. ACCEPTANCE CRITERIA

8.3.1. Negative control

The absolute OD of the negative control (distilled water) is an indicator of tissue viability obtained in the testing laboratory after shipping and storing procedures, and under specific conditions of use. The negative control has to be tested for each run and needs to fall within the range below:

0.7 ≤ mean OD (A570/650) measured value ≤ 2.5

This acceptance range was established from historical quality control data, and consideration reflecting both the shipment and test procedure stresses.

8.3.2. Positive control

The positive control (8N KOH) is tested concurrently with test chemicals in each run, but not more than one positive control is required per testing day. The positive control data result must meet the following condition after 60 minutes exposure:

8N KOH mean tissue viability ≤ 15%

8.3.3. Standard deviation

Since skin corrosion potential is predicted from the mean viability of two individual tissues, the variability of tissue replicated must be kept at an acceptably low level, as stated below:

In the viability range of 20-100%, and ODs ≥ 0.3, the difference of viability between the two replicates should not exceed 30%

8.4. APPLICABILITY DOMAIN AND LIMITATIONS

One limitation of this assay method is a possible interference of the test substance with the MTT endpoint. A colored test substance or one that directly reduces MTT (and thereby mimics dehydrogenase activity of the cellular mitochondria) may interfere with the MTT endpoint. However, these test substances are a problem only if at the time of the MTT test (i.e. after test substance exposure) sufficient amounts of the test substance are still present on (or in) the tissues. In case of this unlikely event, the (true) metabolic MTT reduction and the contribution by a colored test material or (false) direct MTT reduction by the test material can be quantified, as described in section 8.1.2.

9. RESULTS

9.1. QUALITY CONTROL OF TISSUE MODELS

The quality control data for the tissue models used in this validation study demonstrated that the tissue viability, as measured by the MTT assay, and barrier function, measured as the IC_{50} after 18 hours treatment with various concentrations of SLS, were stable among the different batches provided to each laboratory. Moreover, upon histological assessment, all batches of LabCyte EPI-MODEL24 showed multiple layers of viable epithelial cells organized under a functional *stratum corneum*. Using these data, the VMT was able to confirm the completeness of the epidermal tissue layers used in this validation study. All batches used passed the manufacturer's model supply criteria for LabCyte EPI-MODEL24, as shown in Appendix 6.

9.2. QUALITY ASSURANCE

Assays and quality assurance were carried out in the principle of GLP, although not all participating laboratories were GLP certified. Raw data and data sheets were reviewed at each laboratory and then verified for errors and omissions by both the data analysis group and the record management group. All raw data and data analysis sheets were pre-checked for quality by each laboratory and then were reviewed by the VMT quality assurance team. The raw data was found to reflect the test results accurately. (Appendices 7 and 8).

No accidents occurred during the course of the validation study, upon completion of which, all residual test chemicals were disposed of in compliance with the rules and regulations of the participating laboratories, and all MSDS were returned to JaCVAM in their sealed envelopes.

All of the accepted data were used by statisticians for the following analysis (Appendix 9).

9.3. TRAINING PHASE

Prior the validation study, the participating laboratories underwent a training phase to ensure that the test protocol and procedures were correctly understood, and that the chosen facilities were capable to run all experiments. For this phase, seven chemicals were selected from the reference chemical list (2), and positive and negative controls were also tested according to the LabCyte EPI-MODEL24 SCT SOP. It is worth noting that the predictive performance of the tests conducted during this training phase was not considered as a criterion to evaluate the participating laboratories.

Negative and positive control results from Lab A and Lab B were within the established acceptance criteria. Lab C, however, recorded a difference in viability of 40.3% for its negative control at 60 min exposure, and therefore, had to repeat the tests (Table 7, Table 8). The seven test chemicals included two 1A chemicals, and four 1B-and-C chemicals, as classified according to the UN GHS. Both 1A chemicals (Bromoacetic acid and Dichloroacetyl chloride), and one 1B-and-C chemical (Glyoxylic acid monohydrate) were correctly classified by all three participant laboratories. Of the remaining 1B-and-C chemicals, Lactic acid was overclassified as 1A by Lab B and Lab C, and Ethanolamine was overclassified as 1A by Lab A. The other two 1B-and-C chemicals, 4-Amino-1,2,4-trizole and Lauric acid were both underclassified as NC by all three participating laboratories (Table 9).

Table 7 - Negative control results from the training phase

	Training		Re-training	
Lab A (3 min)	0.96 (6.8)	Adapted		
Lab A (60 min)	1.03 (6.1)	Adapted		
Lab B (3 min)	0.81 (9.8)	Adapted		
Lab B (60 min)	1.07 (1.51)	Adapted		
Lab C (3 min)	1.23 (18.8)	Adapted	1.41 (19.4)	Adapted
Lab C (60 min)	0.91 (40.3)	Not adapted	1.50 (5.2)	Adapted

Upper row: OD, lower row (in brackets): difference in viability in %

Table 8 - Positive control results from the training phase

	Training	Re-training
Lab A	1.03 (6.1)	
Lab B	1.27 (0.98)	
Lab C	Not Adapted	0.32 (0.22)

Upper row: cell viability in %, lower row (in brackets): difference in viability in %

Table 9 -SCT results from training phase

Chemical	CAS number	GHS class	State	Lab A Training Viability (%)			Lab B Training Viability (%)			Lab C Re-training Viability (%)		
				3'	60'	Class	3'	60'	Class	3'	60'	Class
Bromoacetic acid	79-08-3	1A	S	0.9	0.4	1A	2.7	1.5	1A	1.4	1.3	1A
Dichloroacetyl chloride	79-36-7	1A	L	0.5	0.2	1A	2.1	1.8	1A	0.5	0.3	1A
Glyoxylic acid monohydrate	563-96-2	1B/C	S	45.6	0.6	1B/C	60.2	3.0	1B/C	74.9	11.0	1B/C
Lactic acid	598-82-3	1B/C	L	28.5	0.7	1B/C	6.6	2.6	1A	12.1	3.8	1A
Ethanolamine	141-43-5	1B/C	L	5.2	5.4	1A	19.1	14.8	1B/C	47.1	1.9	1B/C
4-Amino-1,2,4-trizole	584-13-4	1B/C	S	87.6	64.2	NC	103.0	86.4	NC	99.1	81.6	NC
Lauric acid	143-07-7	1B/C	L	99.6	97.2	NC	108.8	107.5	NC	112.3	102.8	NC

9.4. NEGATIVE CONTROL

Table 7 shows absorbance values for the negative control. All data (11 test runs for three minutes exposure and 11 test runs for 60 minutes exposure) for the negative control met the acceptance criteria for both the OD range (0.7 ≤ Mean OD ≤ 2.5) and difference of viability (≤ 30%). The frequency of invalid test runs for the negative control was 0%.

9.5. POSITIVE CONTROL

Table 8 shows cell viability values for the positive control. All data (11 test runs) for the positive control met the acceptance criteria for both cell viability (≤ 15%) and difference of viability (≤ 30%). The frequency of invalid test runs for the positive control was 0%.

Table 10 - Absorbance and the difference of viability of negative control

	Run 1	Run 2	Run 3	Re-test
Lab A	0.83	0.95	1.02	
(3 min)	0.24	3.48	1.28	
Lab A	0.83	0.91	1.00	
(60 min)	1.93	3.19	4.30	
Lab B	0.99	0.99	1.12	1.09
(3 min)	4.93	5.08	1.01	7.74
Lab B	0.98	1.04	0.97	1.08
(60 min)	2.16	2.90	1.29	1.85
Lab C	1.07	1.27	1.66	1.31
(3 min)	16.20	2.45	3.43	5.45
Lab C	1.02	1.27	1.41	1.31
(60 min)	12.22	3.44	7.43	5.56

Upper row: OD; lower row: difference of viability in %

Table 11 - Cell viability and the difference of viability of positive control

	Run 1	Run 2	Run 3	Re-test
Lab A	0.24	0.11	0.45	
	0.24	0.00	0.50	
Lab B	0.05	0.18	0.00	0.10
	0.10	0.37	0.00	0.02
Lab C	0.70	0.38	0.81	0.57
	1.40	0.20	0.07	0.17

Upper row: Cell viability; lower row: the difference of viability

9.6. PREDICTING THE SKIN CORROSION POTENTIAL OF THE SELECTED CHEMICALS

The results of chemical interference detection with MTT endpoints are shown in the Table 9. Substances No.3, No.14, No.19, No.20, and No.30 were detected as chemicals with MTT reduction potential in all sets of coded chemicals, at all three participating laboratories. Moreover, substance No. 26 was detected as a potential MTT reducer in all sets of coded

chemicals in Lab A and Lab C. The OD values of these chemicals were corrected using freeze-killed tissues at each participating laboratory. No substances were detected as coloring chemicals.

Annex 2 shows the tissue mean viability for each test chemical. The data from Lab A met the acceptance criteria for difference of viability (≤ 30%), and the frequency of invalid test runs was 0% (0/90). On the other hand, Lab B and Lab C had one chemical each (substance No. 22 and No. 12, respectively) showing a difference of viability > 30%, and therefore failing to meet the data acceptance criteria. These data sets were submitted as complete data matrices upon re-testing. Thus, the frequency of invalid test runs at both Lab B and Lab C was 1% (1/91).

Table 12 - Detection of MTT assay interference (coloring material and/or MTT reducer)

No.	UN GHS *in vivo* Cat.	Lab A			Lab B			Lab C		
		Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
1	No Corrosive	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	No Corrosive	ND	ND	ND	ND	ND	ND	ND	ND	ND
3	No Corrosive	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R
4	No Corrosive	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	No Corrosive	ND	ND	ND	ND	ND	ND	ND	ND	ND
6	No Corrosive	ND	ND	ND	ND	ND	ND	ND	ND	ND
7	No Corrosive	ND	ND	ND	ND	ND	ND	ND	ND	ND
8	No Corrosive	ND	ND	ND	ND	ND	ND	ND	ND	ND
9	No Corrosive	ND	ND	ND	ND	ND	ND	ND	ND	ND
10	No Corrosive	ND	ND	ND	ND	ND	ND	ND	ND	ND
11	Cat. 1B and 1C	ND	ND	ND	ND	ND	ND	ND	ND	ND
12	Cat. 1B and 1C	ND	ND	ND	ND	ND	ND	ND	ND	ND
13	Cat. 1B and 1C	ND	ND	ND	ND	ND	ND	ND	ND	ND
14	Cat. 1B and 1C	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R
15	Cat. 1B and 1C	ND	ND	ND	ND	ND	ND	ND	ND	ND
16	Cat. 1B and 1C	ND	ND	ND	ND	ND	ND	ND	ND	ND
17	Cat. 1B and 1C	ND	ND	ND	ND	ND	ND	ND	ND	ND
18	Cat. 1B and 1C	ND	ND	ND	ND	ND	ND	ND	ND	ND
19	Cat. 1B and 1C	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R
20	Cat. 1B and 1C	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R
21	Cat. 1A	ND	ND	ND	ND	ND	ND	ND	ND	ND
22	Cat. 1A	ND	ND	ND	ND	ND	ND	ND	ND	ND
23	Cat. 1A	ND	ND	ND	ND	ND	ND	ND	ND	ND
24	Cat. 1A	ND	ND	ND	ND	ND	ND	ND	ND	ND
25	Cat. 1A	ND	ND	ND	ND	ND	ND	ND	ND	ND
26	Cat. 1A	MTT R	MTT R	MTT R	ND	ND	ND	MTT R	MTT R	MTT R
27	Cat. 1A	ND	ND	ND	ND	ND	ND	ND	ND	ND
28	Cat. 1A	ND	ND	ND	ND	ND	ND	ND	ND	ND
29	Cat. 1A	ND	ND	ND	ND	ND	ND	ND	ND	ND
30	Cat. 1A	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R	MTT R

Abbreviations: NC = Non corrosive; 1B/C: Sub-categories 1B-and-1C; 1A = Sub-category 1A; MTT R = MTT reducer; ND = Not detected.

Table 13 - Mean cell viability and within-laboratory reproducibility (WLR)

No.	UN GHS *in vivo* Cat.	Lab A				Lab B				Lab C			
		Set 1	Set 2	Set 3	WLR	Set 1	Set 2	Set 3	WLR	Set 1	Set 2	Set 3	WLR
1	NC	NC	NC	NC	C	NC	NC	NC	C	NC	NC	NC	C
2	NC	NC	NC	NC	C	NC	NC	NC	C	NC	NC	NC	C
3	NC	NC	NC	NC	C	NC	NC	NC	C	NC	NC	NC	C
4	NC	NC	NC	NC	C	NC	NC	NC	C	NC	NC	NC	C
5	NC	NC	NC	NC	C	NC	NC	NC	C	NC	NC	NC	C
6	NC	NC	NC	NC	C	NC	NC	NC	C	NC	NC	NC	C
7	NC	NC	NC	NC	C	NC	NC	NC	C	NC	NC	NC	C
8	NC	NC	NC	NC	C	NC	NC	NC	C	NC	NC	NC	C
9	NC	1A	1A	1A	C	1A	1A	1A	C	1B/C	1B/C	1B/C	C
10	NC	1B/C	NC	1B/C	N	NC	NC	NC	C	NC	NC	1B/C	N
11	Cat. 1B/C	1B/C	1B/C	1A	N	1A	1B/C	1B/C	N	1B/C	1B/C	1B/C	C
12	Cat. 1B/C	1A	1A	1A	C	1A	1A	1A	C	1A	1A	1B/C	N
13	Cat. 1B/C	1B/C	1B/C	1B/C	C	1B/C	1B/C	1B/C	C	1B/C	1B/C	1B/C	C
14	Cat. 1B/C	1B/C	1B/C	1B/C	C	1B/C	1B/C	1B/C	C	1B/C	1B/C	1B/C	C
15	Cat. 1B/C	1B/C	1B/C	1B/C	C	1B/C	1B/C	1B/C	C	1B/C	1B/C	1B/C	C
16	Cat. 1B/C	1A	1A	1A	C	1A	1A	1A	C	1A	1A	1A	C
17	Cat. 1B/C	1A	1A	1A	C	1A	1A	1A	C	1A	1A	1A	C
18	Cat. 1B/C	1A	1A	1A	C	1A	1A	1A	C	1A	1A	1A	C
19	Cat. 1B/C	1A	1B/C	1A	N	1B/C	1B/C	1B/C	C	1A	1A	1A	C
20	Cat. 1B/C	1A	1A	1A	C	1A	1A	1A	C	1A	1A	1A	C
21	Cat. 1A	1A	1A	1A	C	1A	1A	1A	C	1A	1A	1A	C
22	Cat. 1A	1A	1A	1A	C	1A	1A	1A	C	1A	1A	1A	C
23	Cat. 1A	1A	1A	1A	C	1A	1A	1A	C	1A	1A	1A	C
24	Cat. 1A	1A	1A	1A	C	1B/C	1B/C	1B/C	C	1B/C	1A	1A	N
25	Cat. 1A	1A	1A	1A	C	1A	1A	1A	C	1A	1A	1A	C
26	Cat. 1A	1A	1A	1A	C	1A	1A	1A	C	1A	1A	1A	C
27	Cat. 1A	1A	1A	1A	C	1A	1A	1A	C	1A	1A	1A	C
28	Cat. 1A	1A	1A	1A	C	1A	1A	1A	C	1A	1A	1A	C
29	Cat. 1A	1A	1A	1A	C	1A	1A	1A	C	1A	1A	1A	C
30	Cat. 1A	1B/C	1B/C	1B/C	C	1B/C	1B/C	1B/C	C	1B/C	1B/C	1B/C	C

Abbreviations: NC = Non corrosive; 1B/C: Sub-categories 1B-and-1C; 1A = Sub-category 1A; WLR = within-laboratory reproducibility; C = Concordance; N = Non-concordance.

9.7. STUDY QUALITY CRITERIA

In this validation study, the LabCyte EPI-MODEL24 SCT was performed on 30 test chemicals at three participating laboratories. The dataset had three complete test sequences for each of the thirty test chemicals at all three participating laboratories. Thus, the target (one complete test sequence for each of the thirty test chemicals at any one of the three participating laboratories) for the study quality criteria was achieved.

All participating laboratories achieved 100% (30/30) complete test sequences, therefore achieving the target (≥90% for each laboratory) for the study quality criteria.

9.8. RELIABILITY

9.8.1. Within-laboratory reproducibility

The OECD TG 431 performance standards (2) describes that within-laboratory reproducibility should be calculated based on the concordance of classifications using only qualified tests obtained with reference chemicals for which at least two qualified tests are available. In this study, all participating laboratories produced three qualified tests for all reference chemicals used (Table 10).

The assessment of within-laboratory reproducibility was based on the concordance of predictions obtained from replicate test runs of 30 test chemicals at each of the participating laboratories. As shown in Table 10, Lab B had one non-concordant prediction (No. 11), which resulted in a within-laboratory reproducibility rate of 96.7%. Lab A and Lab C had three non-concordant predictions each (No. 10, No. 11, No. 19 and No. 10, No. 12, No. 24, respectively), and both achieved a within-laboratory reproducibility rate of 90%. Thus, the ≥ 80% target for within-laboratory reproducibility was achieved at each participating laboratory.

9.8.2. Between-laboratory reproducibility

The OECD TG 431 performance standards (2) describes that for the calculation of between-laboratory reproducibility, the final classification for each reference chemical in each participating laboratory should be obtained by using the arithmetic mean value of viability over the different qualified tests performed. It also describes that between-laboratory reproducibility should be calculated based on the concordance of classifications using only qualified tests from reference chemicals for which at least one qualified test per laboratory is available. In this study, all participating laboratories produced three qualified tests for all reference chemicals used.

The assessment of between-laboratory reproducibility was based on the concordance of predictions obtained from replicate test runs of 30 test chemicals at each participating laboratory. As shown in Table 11, between-laboratory reproducibility was 83.3%, as there were four non-concordant predictions (No. 9, No. 10, No. 12, No. 19, No. 24). Nevertheless, the ≥ 70% target for between-laboratory reproducibility was achieved.

Table 14 - Mean cell viability of three independent tests runs and between-laboratory reproducibility (BLR) for LabCyte EPI-MODEL24 SCT

No.	UN GHS *in vivo* Cat.	Lab A			Lab B			Lab C			BLR
		3 min	60 min	Judge	3 min	60 min	Judge	3 min	60 min	Judge	
1	NC	104.2 (7.9)	115.6 (10.0)	NC	115.0 (9.0)	116.8 (11.5)	NC	98.4 (3.4)	122.8 (6.4)	NC	C
2	NC	93.8 (2.1)	71.9 (5.4)	NC	98.4 (14.2)	79.0 (6.0)	NC	93.2 (10.4)	94.3 (8.9)	NC	C
3	NC	101.7 (4.0)	104.5 (7.7)	NC	108.9 (15.3)	112.8 (15.2)	NC	113.3 (18.1)	116.1 (9.2)	NC	C
4	NC	98.4 (2.7)	110.9 (3.9)	NC	102.6 (3.5)	103.3 (5.1)	NC	97.7 (2.1)	116.0 (8.1)	NC	C
5	NC	92.4 (3.9)	107.8 (3.8)	NC	97.3 (2.5)	102.6 (7.8)	NC	106.8 (1.9)	116.1 (8.1)	NC	C
6	NC	65.6 (5.4)	19.7 (1.1)	NC	76.3 (10.0)	21.4 (2.4)	NC	76.0 (2.6)	22.1 (1.8)	NC	C
7	NC	98.9 (2.2)	101.0 (4.8)	NC	99.5 (6.8)	98.1 (3.1)	NC	98.1 (1.6)	109.8 (9.0)	NC	C
8	NC	101.6 (12.4)	103.6 (10.7)	NC	103.3 (8.7)	99.7 (5.1)	NC	102.1 (11.9)	110.7 (9.4)	NC	C
9	NC	5.1 (1.6)	5.6 (1.9)	1A	6.5 (1.0)	8.6 (5.6)	1A	47.4 (15.2)	4.9 (4.3)	1B/C	N
10	NC	64.7 (6.1)	13.4 (2.2)	1B/C	77.4 (14.3)	17.9 (0.4)	NC	104.7 (6.8)	15.2 (0.5)	NC	N
11	1B/C	15.1 (1.8)	0.4 (0.2)	1B/C	31.0 (30.0)	2.2 (0.3)	1B/C	56.1 (12.5)	0.9 (0.5)	1B/C	C
12	1B/C	3.8 (1.3)	2.7 (1.9)	1A	6.6 (1.1)	2.8 (1.5)	1A	23.9 (34.1)	0.4 (0.5)	1B/C	N
13	1B/C	47.3 (3.3)	1.6 (0.8)	1B/C	68.4 (2.0)	2.7 (1.1)	1B/C	76.5 (3.9)	4.7 (1.2)	1B/C	C
14	1B/C	21.1 (2.1)	3.6 (2.3)	1B/C	37.1 (14.6)	6.1 (3.0)	1B/C	59.9 (6.6)	8.1 (3.6)	1B/C	C
15	1B/C	44.1 (28.1)	4.6 (0.2)	1B/C	26.3 (6.9)	3.7 (2.3)	1B/C	31.5 (10.9)	7.5 (5.9)	1B/C	C
16	1B/C	2.8 (1.0)	0.8 (0.1)	1A	4.0 (1.5)	2.2 (0.7)	1A	5.5 (2.0)	2.3 (1.0)	1A	C
17	1B/C	0.6 (0.1)	0.4 (0.3)	1A	2.7 (0.8)	1.9 (0.9)	1A	0.8 (0.4)	0.3 (0.2)	1A	C
18	1B/C	1.4 (0.2)	1.4 (0.5)	1A	2.4 (0.2)	2.6 (0.5)	1A	2.0 (0.6)	1.2 (0.5)	1A	C
19	1B/C	13.1 (7.3)	7.1 (7.0)	1A	24.2 (8.6)	10.8 (8.1)	1B/C	8.5 (2.1)	5.5 (9.6)	1A	N
20	1A	0.7 (0.2)	0.4 (0.2)	1A	4.0 (2.3)	4.6 (2.4)	1A	1.3 (0.1)	2.4 (2.1)	1A	C
21	1A	0.6 (0.2)	1.6 (1.3)	1A	2.4 (0.5)	2.6 (1.1)	1A	0.6 (0.0)	1.4 (0.7)	1A	C
22	1A	0.8 (0.2)	0.5 (0.2)	1A	1.3 (0.6)	1.5 (0.6)	1A	0.8 (0.6)	0.6 (0.4)	1A	C
23	1A	7.6 (0.7)	1.3 (0.4)	1A	8.8 (3.5)	1.9 (0.8)	1A	6.9 (1.9)	3.2 (2.1)	1A	C
24	1A	13.2 (1.6)	6.4 (1.8)	1A	18.5 (3.0)	10.1 (0.4)	1B/C	12.9 (2.8)	8.6 (1.7)	1A	N
25	1A	2.1 (1.5)	0.2 (0.1)	1A	2.8 (1.2)	1.1 (0.3)	1A	0.8 (0.6)	0.3 (0.3)	1A	C
26	1A	0.0 (0.0)	0.0 (0.1)	1A	1.8 (0.6)	2.3 (0.7)	1A	1.1 (0.9)	0.6 (0.6)	1A	C
27	1A	0.3 (0.1)	0.2 (0.2)	1A	1.2 (0.5)	1.2 (0.3)	1A	0.0 (0.1)	0.1 (0.1)	1A	C
28	1A	0.4 (0.2)	0.3 (0.1)	1A	1.1 (0.4)	0.9 (0.3)	1A	0.3 (0.2)	0.1 (0.2)	1A	C
29	1A	0.2 (0.2)	0.2 (0.2)	1A	3.4 (1.9)	1.0 (0.4)	1A	1.5 (1.0)	0.4 (0.2)	1A	C
30	1A	39.7 (4.1)	1.8 (0.3)	1B/C	39.9 (19.8)	3.7 (0.8)	1B/C	64.4 (8.8)	5.5 (0.7)	1B/C	C

Right side: viability in %, Left side (in brackets): difference in viability in %.
Abbreviations: NC = Not corrosive; 1B/C: Sub-categories 1B-and-1C; 1A = Sub-categories 1A; BLR = Between-Laboratory Reproducibility; C = Concordance; N = Non-concordance.

9.9. PREDICTIVE CAPACITY

Predictive capacity was assessed based on the concordance between the predictions made with the data obtained during this validation study with the *in vivo* categories specified in the performance standards for TG 431 (2). The 30 reference chemicals included 10 UN GHS non-corrosive chemicals, 10 chemicals that were either UN GHS sub-categories 1B or 1C, and 10 UN GHS sub-category 1A chemicals, to determine whether proposed test methods support sub-categorization of corrosive chemicals. The minimum predictive capacity values that should be obtained with these reference chemicals are shown in Table 5. The LabCyte EPI-MODEL 24 SCT target values were determined based on the required predictive capacity established in the OECD TG 431 performance standard and on LabCyte EPI-MODEL 24 SCT historical data (underlined chemicals in Annex 1, Annex 2, and Table 5). Moreover, according to the performance standards, a similar or modified RhE test method may be considered similar to EpiSkin™ or to EpiDerm™, depending on the results obtained with the reference chemicals. As shown in Table 5, LabCyte EPI-MODEL 24 SCT is considered similar to EpiDerm™.

Out of 30 test chemicals, Lab A, B, and C correctly predicted 27, 24, and 26 UN GHS sub-category 1A chemicals, respectively, resulting in a correct prediction rate of 85.6%. Incorrectly classified sub-category 1A chemicals were all underclassified as 1B-and-1C chemicals, with no 1A chemicals being underclassified as NC. Altogether, all participant laboratories met the predictive capacity requirements related to the sub-classification of 1A chemicals, according to the TG 431 performance standards. On the other hand, none of the laboratories met the predictive capacity requirements for the sub-categories 1B-and-1C, with only 12, 14, and 13 chemicals correctly classified by Lab A, B, and C, respectively. The remaining chemicals were all overclassified as sub-category 1A. Overall, the rate of correctly classified 1B-and-1C chemicals was 43.3%, and the rate of 1B-and-1C chemicals overclassified

as 1A was 56.7%. It is worth noting that no false negatives were generated by the participant laboratories, therefore meeting the predictive capacity requirements of 0% for 1A chemicals underclassified NC, and ≤ 5% for 1B-and-1C chemicals underclassified as NC.

False positives were obtained by all three participating laboratories. Test chemical No. 9, 2-Hydroxyiso-butyric acid, was overclassified by Lab A and B as sub-category 1A, and by Lab C as a sub-category 1B-and-1C chemical. Test chemical No. 10, Sodium undecylenate (33%), was overclassified as 1B-and-1C sub-category by Lab A and Lab C in two and one out of three runs, respectively.

As a result, the calculated accuracy for Lab A was 71.1%, and for both Lab B and C, 72.2%. Overall, the total accuracy was 71.8 %, thus not meeting the required predictive capacity of ≥ 72% established by the TG 431 performance standards. The results above are summarized in Table 12 (3x3 tables by laboratory) and Table 13 (cumulative 3x3 table for all laboratories). Table 14 shows the predictive capacity rates by laboratory, and the total rate for the entire validation study.

Table 15 - 3x3 tables for each participant laboratory

Lab A		LabCyte EPI-MODEL24 SCT			
		1A	1B/C	NC	Total
in vivo class	1A	27	3	0	30
	1B/C	18	12	0	30
	NC	3	2	25	30
Total		48	17	25	90

Lab B		LabCyte EPI-MODEL24 SCT			
		1A	1B/C	NC	Total
in vivo class	1A	24	6	0	30
	1B/C	16	14	0	30
	NC	3	0	27	30
Total		43	20	27	90

Lab C		LabCyte EPI-MODEL24 SCT			
		1A	1B/C	NC	Total
in vivo class	1A	26	4	0	30
	1B/C	17	13	0	30
	NC	0	4	26	30
Total		43	21	26	90

Table 16 - 3x3 cumulative table for all participating laboratories

All labs		LabCyte EPI-MODEL24 SCT			
		1A	1B/C	NC	Total
in vivo class	1A	77	13	0	90
	1B/C	51	39	0	90
	NC	6	6	78	90
Total		134	58	78	270

Table 17 - Predictive capacity results by laboratory

	Lab A	Lab B	Lab C	All Labs	LabCyte EPI-MODEL24 requirements
Sensitivity (for predictions C vs. NC)	100%	100%	100%	100%	≥ 95.0%
Correctly classified 1A	90.0%	80.0%	86.7%	85.6%	≥ 80.0%
1A underclassified 1B-and-1C	10.0%	20.0%	13.3%	14.4%	≤ 20.0%
1A underclassified NC	0%	0%	0%	0%	0%
Correctly classified 1B-and-1C	***40.0%***	***46.7%***	***43.3%***	***43.3%***	≥ 55.0%
1B-and-1C overclassified 1A	***60.0%***	***53.3%***	***56.7%***	***56.7%***	≤ 45.0%
1B-and-1C underclassified NC	0%	0%	0%	0%	≤ 5.0%
Specificity	83.3%	90.0%	86.7%	86.7%	≥ 70.0%
NC overclassified 1A	***10.0%***	***10.0%***	0%	***6.7%***	≤ 5.0%
NC overclassified 1B-and-1C	6.7%	0%	13.3%	6.7%	≤ 30.0%
Accuracy (C vs. NC)	94.4%	96.7%	95.6%	95.6%	≥ 87.0%
Accuracy (1A vs. 1B-and-1C vs. NC)	***71.1%***	72.2%	72.2%	***71.9%***	≥ 72.0%

Numbers in italic: rates that do not meet the required values.

9.10. ADDITIONAL EXPERIMENTS PERFORMED BY THE LEAD LABORATORY

Considering LabCyte EPI-MODEL's low sub-categorization performance during this validation study (Table 14), the VMT requested the lead laboratory to re-test some of the substances that were either over- or underclassified by the participating laboratories, and differently classified by the lead laboratory (historical data, Annex 1). Substances No. 9, 10, 11, 12, 16, and 24 were therefore coded by the chemical management group, and delivered to the lead laboratory, which performed one test run for each these chemicals.

The lead laboratory accurately reproduced the historical data of all test chemicals (Table 15). Furthermore, chemicals No. 10, 11, 12, 16 were correctly sub-categorized, with results matching the GHS classification (Table 15).

Because test chemical No. 16 (14.4% hydrochloric acid) was the only substance none of the

participating laboratories were capable to correctly classify, the lead laboratory also tested 10% and 18% hydrochloric acid solutions to assess LabCyte EPI-MODEL responsiveness to this particular substance. LabCyte showed a linear response to different concentrations of hydrochloric acid after three minutes exposure (Figure 3, Annex 1). Although this responsiveness was less pronounced after 60 minute exposure, 10% and 14.4% solutions were properly classified as 1B-and-1C, and the 18% solution was classified as 1A.

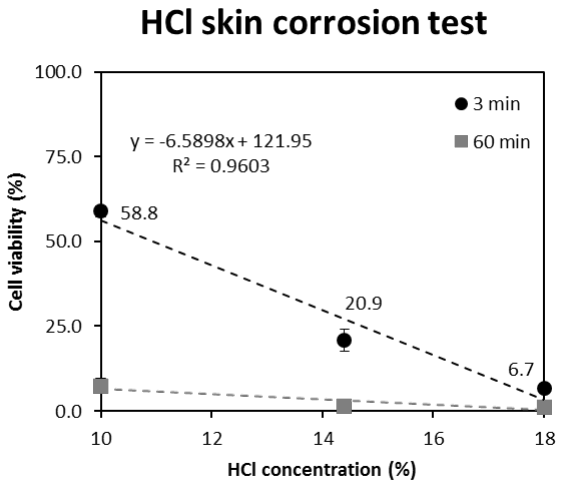

Figure 4 - Skin corrosion test of hydrochloric acid

Table 18 - Addtional tests conduced by the lead laboratory

No.	Chemical	GHS class		Additional Test by Lead Lab	Validation Study									Historical data[1]		
					Lab A			Lab B			Lab C			Lead Lab		
					1	2	3	1	2	3	1	2	3	1	2	3
9	2-Hydroxyiso-butiric acid	N	3 min 60 min Judge	35.4% 12.6% 1B/C	*4.8%* *3.9%* *1A*	*6.8%* *7.6%* *1A*	*3.7%* *5.2%* *1A*	*5.7%* *12.7%* *1A*	*7.6%* *10.9%* *1A*	*6.3%* *8.9%* *1A*	30.0% 0.3% 1B/C	58.0% 5.4% 1B/C	54.3% 12.6% 1B/C	68.5% 14.4% 1B/C	61.7% 7.4% 1B/C	43.3% 10.6% 1B/C
10	Sodium undecylenate	N	3 min 60 min Judge	**64.8%** **17.2%** **N**	63.3% 13.3% 1B/C	**71.4%** **15.7%** **N**	59.5% 11.3% 1B/C	**86.0%** **18.2%** **N**	**60.9%** **18.1%** **N**	**85.4%** **17.4%** **N**	**104.4%** **15.5%** **N**	**111.6%** **15.5%** **N**	96.1% 14.7% 1B/C	90.0% 13.9% 1B/C	**97.8%** **19.9%** **N**	**81.2%** **20.4%** **N**
11	Glycoxylic acid monohydrate	1B/C	3 min 60 min Judge	**71.6%** **1.3%** **1B/C**	**16.7%** **0.2%** **1B/C**	**15.4%** **0.6%** **1B/C**	13.2% 0.4% A	12.2% 2.5% A	**15.2%** **2.0%** **1B/C**	**65.6%** **2.0%** **1B/C**	**41.6%** **0.4%** **1B/C**	**63.4%** **0.7%** **1B/C**	**63.2%** **1.5%** **1B/C**	**77.6%** **1.9%** **1B/C**	**71.5%** **0.5%** **1B/C**	**62.9%** **0.4%** **1B/C**
12	Lactic acid	1B/C	3 min 60 min Judge	**59.6%** **0.4%** **1B/C**	5.2% 0.5% 1A	3.5% 4.0% 1A	2.6% 3.5% 1A	5.8% 1.4% 1A	6.1% 4.4% 1A	7.8% 2.6% 1A	0.0% 0.0% 1A	8.7% 0.9% 1A	**62.9%** **0.4%** **1B/C**	**58.2%** **8.4%** **1B/C**	**52.1%** **1.0%** **1B/C**	**59.5%** **0.9%** **1B/C**
16	Hydrochloric acid	1B/C	3 min 60 min Judge	**26.1%** **0.8%** **1B/C**	3.9% 0.9% 1A	2.4% 0.7% 1A	2.1% 0.6% 1A	4.1% 2.8% 1A	2.5% 1.7% 1A	5.4% 2.2% 1A	3.3% 3.4% 1A	6.0% 1.8% 1A	7.3% 1.7% 1A	**17.4%** **1.9%** **1B/C**	**21.6%** **1.0%** **1B/C**	**23.6%** **1.3%** **1B/C**
24	Phenol	1A	3 min 60 min Judge	15.0% 10.7% 1B/C	**14.4%** **7.6%** **1A**	**13.9%** **7.2%** **1A**	**11.4%** **4.3%** **1A**	21.3% 9.7% 1B/C	18.8% 10.1% 1B/C	15.4% 10.5% 1B/C	15.5% 10.5% 1B/C	**13.1%** **7.3%** **1A**	**10.0%** **8.1%** **1A**	27.9% 22.1% 1B/C	**13.8%** **11.4%** **1A**	16.9% 11.2% 1B/C

[1]Test of 79 substances performed by the lead laboratory (Annex 1).

10. DISCUSSION

10.1. IN CONSIDERATION OF INVALID RESULTS

As all test runs for negative and positive controls met the acceptance criteria, the VMT concluded that these results were highly reproducible over the duration of this validation study (Table 7 and Table 8). Although Lab B and Lab C produced one invalid run each, complete data matrices were submitted upon re-testing. Finally, the data of this validation study met the study quality criteria according to the OECD TG 431 performance standard.

10.2. RELIABILITY

Within-laboratory reproducibility was 90% at Lab A, 96.7% at Lab B, and 90.0% at Lab C, thus achieving the ≥80% requirement specified in the TG 431 performance standards (2). Moreover, between-laboratory reproducibility for all three participating laboratories was 83.3%, thus achieving the ≥70% requirement specified in the TG 431 performance standards (2). These results demonstrate that the robustness and reliability of the LabCyteEM24 SCT method are sufficient to meet the requirements of the performance standards.

10.3. PREDICTIVE CAPACITY

The LabCyte EPI-MODEL24 SCT is undoubtedly able to distinguish corrosive from non-corrosive substances, as sensitivity was 100% across all three laboratories, meeting the established test requirements (Table 14). Overall accuracy and specificity were 95.6% and 86.7%, respectively, also meeting the established targets and demonstrating that LabCyte EPI-MODEL24 is indeed useful for the identification of corrosive substances. As a test method used for the sub-categorization of corrosives into sub-category 1A, LabCyte EPI-MODEL24 also

performed well at all laboratories, with an overall rate of 85.6% of correctly classified 1A substances. Similarly to the VRMs, substance No. 30 was underclassified as sub-category 1B-or-1C.

The performance of sub-categorization of corrosives into a combination of sub-categories 1B and 1C, however, did not meet the target requirements. Six 1B-and-1C substances (No. 12, 16, 17, 18, 19, 20) were overclassified as 1A, and substance No. 11 (1B or 1C) had two runs that resulted in 1A. Overall, the rate of correctly classified 1B-and-1C substances was 43.3%, which in turn led to a rate of 56.7% of 1B-and-1C substances overclassified 1A. Compared to the VRMs, only the LabCyte EPI-MODEL24 SCT overclassified substances No. 12 and 16. However, both VRMs overclassified substances No. 17 and 18 (2). Moreover, EpiDerm™ SCT also overclassified substances No. 19 and 20, suggesting that these chemicals cannot be easily sub-categorized by the TG 431.

Two false positives were detected during the validation study, as substances No. 9 and 10 were overclassified as sub-category 1A and 1B-and-1C, respectively. Overclassification of substance No. 9 resulted in a 6.7% ratio of NC overclassified 1A substances, not meeting the target requirement of ≤ 5.0% (Table 14). Overclassification of substance No. 10 has also been reported from other VRMs (2).

To address this sub-categorization issue, the lead laboratory re-tested substances No. 9, 10, 11, 12, 16, 24, and was able to reproduce the in-house data acquired previously (Annex 1, Table 15). The results obtained by the lead laboratory for substances No. 9, 10, 11, and 24 corresponded to, as least in part, those obtained by each facility, and fell in the range of cell viability data recorded during this validation study. The reasons for the partial discrepancy between these results are unknown. However, as the results for substance No. 12 at Lab C showed (Annex 2, Table 15), the variability between test runs can be high, especially if the

chemical results in cell viability are close to 50% after three minutes exposure. High variability is also observed in in-house results for substance No. 16 (Annex 1), the only re-tested chemical that none of the participating laboratories predicted correctly. Although this is also representative of the LabCyte EPI-MODEL tendency to overclassify 1B-and-1C chemicals into 1A, the lead laboratory tested hydrochloric acid at different concentrations to assess LabCyte EPI-MODEL responsiveness to this particular substance. Although the model's reaction to different concentrations of hydrochloric acid was less pronounced after 60 minutes exposure, a linear response was observed at after three minutes exposure. This suggests that LabCyte EPI-MODEL can indeed detect these concentration differences and can be used to predict the corrosive potential of hydrochloric acid, even though all three participant laboratories overclassified it as a category 1A chemical.

Sensitivity at all participating laboratories individually, as well as collectively, was 100%. Correctly classified 1A substances ranged from 80 to 90% across laboratories. Likewise, specificity ranged from 83.3 to 86.7%, and accuracy (C vs. NC) from 94.4 to 96.7%, thus meeting the established requirements (Table 14). Accuracy (1A vs. 1B-and-1C vs. NC) ranged from 71.1 to 72.2%, and did not fulfill the requirement. Altogether, these results suggest that LabCyte EPI-MODEL24 has the tendency to overclassify 1B-and-1C chemicals, and this tendency became evident during the validation study.

The reasons for the difference in performance observed between this validation study and the historical data can only be speculated. Since the training phase was successfully conducted at the three participant laboratories, it is highly unlikely that technical skills-related issues caused the discrepancy between the results. Even though the test substances were carefully washed and removed after exposure, there is a chance that chemical residues within the tissue caused the variability in cell viability, particularly during the post-exposure incubation time.

Nevertheless, as the additional experiments performed by the lead laboratory indicated, LabCyte EPI-MODEL24 can be used to sub-categorize corrosive chemicals into 1A and a combination of 1B-and-1C chemicals.

10.4. EVALUATION OF THE PREDICTION MODEL

The LabCyte EPI-MODEL24 SCT prediction model was established based on the EpiDerm™ SCT (EPI-200), in addition to analysis of historical data. The cutoff (cell viability) values were set so false negatives were not generated, and false positives were minimized. Considering the sub-classification performance of LabCyte EPI-MODEL24 SCT during the validation study, the prediction model was re-evaluated using different cutoff values and through receiver operating characteristic (ROC) analysis.

The sub-classification of corrosive chemicals is done based on the cell viability at 3 minutes exposure. Table 16 shows the sub-classification performance of 79 substances tested in-house, using different cutoff values. It is worth noting that any cutoff value below 12% generates false negatives. The ROC analysis accompanying the cutoff value test is shown in Table 19 and Figure 5.

Table 19 - Cutoff value test using historical data (79 substances)

Cutoff value	Correct 1B-and-C	Correct 1A
12%	72%	78%
13%	71%	81%
14%	68%	83%
15%	68%	86%
16%	68%	86%
17%	68%	89%
18%	62%	89%

The same test was performed with the validation test results (Table 17).

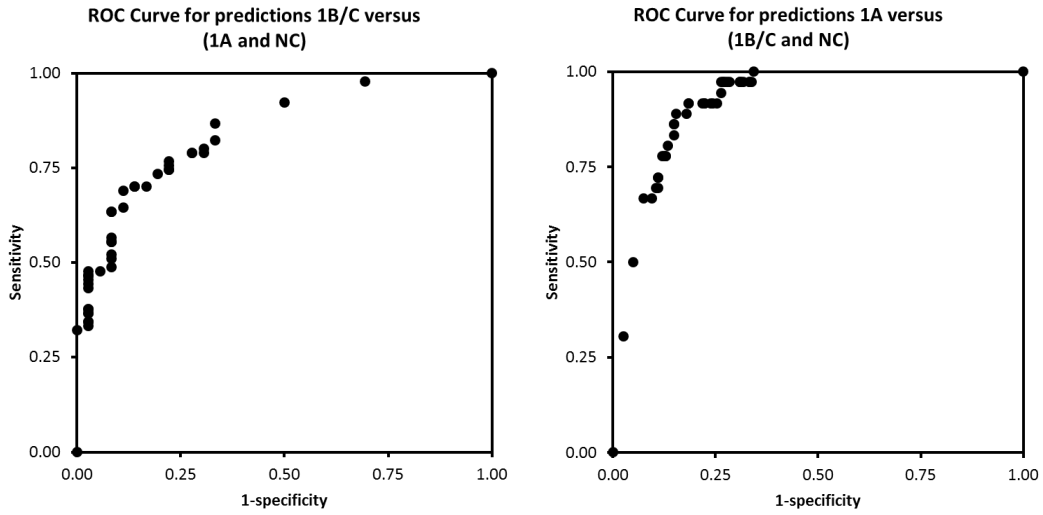

Figure 5 - ROC analysis of in-house database chemicals (79 chemicals)

Although the historical data test (Table 16) suggests that a cutoff of 17% for 3 minute exposure might improve the prediction model, the validation study results demonstrate that such cutoff would have led to an even poorer classification of 1B-and-C chemicals, at all three participant laboratories.

Table 20 - Cutoff value test using validation study data

Cutoff value	Correctly classified 1B-and- C			Correctly classified 1A		
	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C
12%	50%	50%	43%	80%	76%	83%
13%	40%	46%	43%	80%	80%	83%
14%	40%	46%	43%	90%	80%	86%
15%	40%	46%	43%	90%	80%	86%
16%	40%	43%	43%	90%	83%	90%
17%	30%	40%	43%	90%	83%	90%
18%	30%	40%	43%	90%	83%	90%

10.5. EFFECT OF TRANSPORTATION TO TISSUE MODELS

Another factor that could have influenced the results of the corrosive chemicals sub-classifications during the validation study is the delivery (transportation) of the tissue models to the participant laboratories. Because in-house data generated at J-TEC did not account for issues occurred during product transportation (i.e. tissues models are manufactured and tested at the same site), it is plausible to think this is the reason why participant laboratories were not able to reproduced J-TEC's historical data.

LabCyte EPI-MODEL have been used outside Japan, and although long-distance shipment may have impacted cell viability and barrier function, the values obtained at the test sites were well within the acceptance criteria established by the manufacturer (Table 18).

Table 21 - Long-distance shipment effect on LabCyte EPI-MODEL

Lot not. ***Destination***	**Tissue viability 570/650 nm OD (criteria: 0.7 – 2.5)**		**Barrier Function SLS IC50 (%) (criteria: 0.14 - 0.4)**	
	Test site result	Batch release QC	Test site result	Batch release QC
LCE24-090824-A ***China***	0.871	1.6	0.32	0.24
LCE24-090831-A ***China***	1.011	1.44	0.27	0.21
LCE24-090914-A ***China***	1.054	1.35	0.25	0.23
LCE24-180326-A ***USA***	0.996	1.2	0.34	0.27

10.6. SIMILARITY WITH THE OECD TG 431

As previously described in section 5, the VMT considers LabCyte EPI-MODEL24 SCT to be functionally similar to the TG 431 VRMs. Using 30 Reference Chemicals from TG 431 as test chemicals, LabCyte EPI-MODEL24 SCT and the VRMs generated two false positives from no

corrosive chemicals, between two to six over-predictions for 1B-and-1C chemicals, and one under-prediction for 1A chemicals. Prediction of the remaining 24 to 26 chemicals was concordant between the LabCyte EPI-MODEL 24 SCT and the VRMs. These results suggest that LabCyte EPI-MODEL24 SCT has a predictive capacity similar to, or higher than, that of the VRMs (Table 18).

10.7. SIMILARITY WITH OECD TG431 VRMS

A more thorough assessment of the predictive capacity of LabCyte EPI-MODEL SCT can be done by comparing its performance to that of the TG 431 VRMs, based on the test of 79 substances (Annex 1, Table 16). Although LabCyte EPI-MODEL SCT showed a tendency to produce false positives, particularly by overclassifying 1B-and-1C chemicals into 1A, this rate (21.5%) was comparable to that of Epiderm (23.3%). Underclassification of substances, on the other hand, was less of an issue as the LabCyte EPI-MODEL SCT underclassification rate was 2.1%, and lower than that of the EpiSkin and Epiderm (3.3% and 2.5%, respectively).

Based on the above, LabCyte EPI-MODEL SCT has a lower rate of correctly classified 1A and 1B-and-1C chemicals, compared to the VRMs (Table 16). However, the rate of correctly classified non-corrosive chemicals and overall accuracy falls between both VRMs, demonstrating that LabCyte EPI-MODEL SCT is indeed a similarly capable method to assess the skin corrosion potential of chemicals.

Table 22 - LabCyte EPI-MODEL24 SCT performance comparison

	LabCyte	VRMs	
	EPI-MODEL24	EpiSkin™	Epiderm™
1A correctly classified	86.1%	83.3%	83.3%
1A underclassified 1B-and-1C	13.9%	16.7%	16.7%
1A underclassified NC	0.0%	0.0%	0.0%
1B-and-/1C correctly classified	70.0%	76.3%	71.0%
1B-and-1C overclassified 1A	30.0%	21.5%	29.0%
1B-and-1C underclassified NC	0.0%	2.2%	0.0%
NC correctly classified (Specificity)	78.4%	79.3%	73.9%
NC overclassified 1A	2.7%	0.0%	2.7%
NC overclassified 1B-and-1C	18.9%	20.7%	23.4%
Overall Accuracy	76.4%	78.8%	74.2%
Global overclassification rate (all categories)	21.5%	17.9%	23.3%
Global underclassification rate (all categories)	2.1%	3.3%	2.5%

Overclassification rates, underclassification rates, and accuracy (predictive capacity) of LabCyte EPI-MODEL24 SCT, based on a set of 79 chemicals tested over three independent runs. VRM results, according to OECD TG431 (3), are provided for comparison.

11. CONCLUSION

This validation study intended to demonstrate that the LabCyte EPI-MODEL24 SCT is capable of fulfilling the performance standards stipulated in OECD TG 431 for similar or modified *in vitro* RhE SCT methods based on the VRMs. The study was designed both to provide the information necessary to validating the test method, as well as to minimize the burden placed on the three participating laboratories.

Having achieved within-laboratory reproducibility from 90.0 to 96.7 % at each of the three participating laboratories, as well as a between-laboratory reproducibility of 83.3 % for all three participating laboratories combined, the validation study was successful and provided evidence that the LabCyte EPI-MODEL24 SCT meets the performance standards for TG 431.

The LabCyte EPI-MODEL24 SCT also demonstrated good predictive capacity with overall correctly classified 1A ratio of 85.6 %, overall specificity of 86.7%, and overall accuracy (C vs. NC) of 95.6 %, thereby meeting the acceptance criteria of 80% for correctly classified 1A, 70% for specificity, and 87% for accuracy (C vs. NC). However, the results of the LabCyte EPI-MODEL24 SCT validation study did not meet the target value of 43.3% of correctly classified 1B-and-1C.

Altogether, these results suggest that the LabCyte EPI-MODEL24 SCT validation study was successfully conducted, and that the test method is a robust and reliable method for predicting the skin corrosion potential of chemicals. Most importantly, the test data provides information that supports the proposal of LabCyte EPI-MODEL24 SCT as a me-too method for inclusion in OECD TG 431.

ACKNOWLEDGMENTS

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ANNEX 1: LABCYTE EPI-MODEL24 SCT PERFORMANCE EVALUATION

The lead laboratory tested 79 substances in order to evaluate the predictive capacity of LabCyte EPI-MODEL24 SCT. The substances used, and the respective corrosion predictions are provided in the table below. OECD TG 431 References chemicals appear underlined.

Chemical	CAS number	State	Run 1			Run 2			Run 3			Final classification
			Viability (%)			Viability (%)			Viability (%)			
			3'	60'	Class	3'	60'	Class	3'	60'	Class	
Non-corrosive chemicals based on vivo results												
o–Methoxyphenol (guaiacol)	90-05-1	L	45.2	37.4	1B/C	36.4	6.3	1B/C	26.9	5.4	1B/C	1B/C
2,4–dimethylaniline	95-68-1	L	75.1	18.2	NC	73.7	28.8	NC	58.3	27.2	NC	NC
Phenethyl bromide	103–63–9	L	123.7	91.1	NC	109.6	124.0	NC	101.2	113.8	NC	NC
Butyl carbamate	592–35–8	S	84.2	37.3	NC	87.9	22.5	NC	90.7	16.4	NC	NC
L–Glutamic acid hydrochloride	138–15–8	S	87.3	32.5	NC	91.2	47.8	NC	95.4	17.6	NC	NC
1–(o–Tolyl)biguanide	93–69–6	S	86.5	37.1	NC	90.1	67.8	NC	97.0	43.0	NC	NC
Butyl glycolate (polysolvan)	7397–62–8	L	73.5	23.9	NC	82.0	21.8	NC	94.8	23.1	NC	NC
2–Hydroxyisobutyiric acid	594–61–6	S	68.5	14.4	1B/C	61.7	7.4	1B/C	43.3	10.6	1B/C	1B/C
Oxalic acid dehydrate	6153–56–6	S	45.6	0.9	1B/C	75.6	1.1	1B/C	90.2	1.0	1B/C	1B/C
Alpha–Ketoglutaric acid	328–50–7	S	20.5	1.4	1B/C	17.4	2.7	1B/C	53.1	2.7	1B/C	1B/C
Sulphamic acid	5329–14–6	S	73.9	2.1	1B/C	68.6	1.7	1B/C	83.3	1.6	1B/C	1B/C
Lauric acid	143–07–7	S	82.3	97.5	NC	118.8	80.7	NC	102.4	86.7	NC	NC
Sodium lauryl sulphate (20%)	151–21–3	L	109.1	28.6	NC	89.6	21.2	NC	104.1	16.5	NC	NC
Methyl trimethylacetate	598–98–1	L	99.7	24.9	NC	111.0	36.9	NC	111.8	38.2	NC	NC
4–Amino–4H–1,2,4–triazole	584–13–4	S	99.6	78.1	NC	97.0	99.5	NC	90.9	76.3	NC	NC
1,9–Decadiene	1647–16–1	L	94.3	93.4	NC	120.6	84.6	NC	97.7	88.5	NC	NC
Sodium carbonate (50%)	497–19–8	L	54.3	29.3	NC	59.1	18.2	NC	56.2	31.8	NC	NC
Benzylacetone (4–phenyl–2–butanone)	2550–26–7	L	118.7	100.4	NC	104.5	99.9	NC	99.6	99.5	NC	NC
Eugenol	97–53–0	L	63.1	20.1	NC	69.9	19.9	NC	46.7	20.9	1B/C	1B/C(1T),NC(2T)
Tetrachloroethylene	127–18–4	L	111.4	75.1	NC	99.2	87.6	NC	105.8	95.7	NC	NC
Sodium undecylenate (33%)	3398–33–2	L	90.0	13.9	1B/C	97.8	19.9	NC	81.2	20.4	NC	1B/C(1T),NC(2T)
4–Amino–5–methoxy–2–methylbenzensulphonic acid	6471–78–9	S	100.6	35.7	NC	110.3	19.1	NC	107.5	90.3	NC	NC

Chemical	CAS number	State	Run 1			Run 2			Run 3			Final classification
			Viability (%)			Viability (%)			Viability (%)			
			3'	60'	Class	3'	60'	Class	3'	60'	Class	
Potassium hydroxide (5%)	1310-58-3	L	1.0	0.4	1A	0.6	0.4	1A	0.3	0.4	1A	1A
3,3-Dithiopropionic acid	1119-62-6	S	101.1	102.6	NC	109.4	88.4	NC	102.1	100.5	NC	NC
Isopropanol	67-63-0	L	94.0	18.1	NC	103.1	31.9	NC	102.3	27.3	NC	NC
2-Phenylalcohol (2-Phenetyl ethanol)	60-12-8	L	91.3	30.4	NC	90.5	19.9	NC	80.8	26.0	NC	NC
n-Buthyl propionate	590-01-2	L	92.8	46.5	NC	99.5	72.6	NC	102.6	88.1	NC	NC
Methyl palmitate	112-39-0	S	95.6	95.0	NC	111.5	89.3	NC	94.7	92.1	NC	NC
Methyl laurate	111-82-0	L	91.1	83.8	NC	87.4	102.1	NC	93.2	94.8	NC	NC
Sodium bicarbonate	144-55-8	S	94.3	85.5	NC	96.1	96.0	NC	99.3	85.2	NC	NC
2-Bromobutane	78-76-2	L	102.1	42.7	NC	115.2	24.7	NC	102.4	47.7	NC	NC
4-(Methylthio)-benzaldehyde	3446-89-7	L	102.1	82.4	NC	89.6	85.4	NC	100.7	85.2	NC	NC
2-Ethoxyethyl methacrylate	2370-63-0	L	84.7	60.8	NC	87.7	82.3	NC	99.1	81.4	NC	NC
Cinnamaldehyde	14371-10-9	L	37.1	12.4	1B/C	82.1	14.3	1B/C	67.7	27.7	NC	1B/C(2T),NC(1T)
4,4´-Methylene-bis-(2,6-ditert-butylphenol)	118-82-1	S	95.8	84.5	NC	98.6	116.9	NC	98.8	90.0	NC	NC
Sodium bisulfite	7631-90-5	S	91.7	8.0	1B/C	83.7	11.1	1B/C	100.5	16.7	NC	1B/C(2T),NC(1T)
10-Undecenoic acid	112-38-9	S	67.0	25.6	NC	65.8	20.1	NC	57.8	26.0	NC	NC
Combination of UN GHS Sub-categories 1B and 1C based on in vivo results												
N,N-Dimethylbenzylamine	103-83-3	L	43.4	3.6	1B/C	33.9	2.8	1B/C	42.6	2.5	1B/C	1B/C
Fluroboric acid	16872-11-0	L	3.8	2.3	1A	0.7	1.6	1A	1.3	0.4	1A	1A
Maleic anhydride	108-31-6	S	79.0	3.0	1B/C	23.9	0.3	1B/C	43.5	0.4	1B/C	1BC
60/40 octanoic/decanoic acid	68937-75-7	L	18.3	7.5	1B/C	63.9	5.3	1B/C	17.3	5.4	1B/C	1BC
55/45 octanoic/decanoic acid	68937-75-7	L	23.4	3.1	1B/C	25.7	5.6	1B/C	33.3	4.3	1B/C	1BC
65/35 octanoic/decanoic acid	68937-75-7	L	23.7	2.0	1B/C	33.0	4.8	1B/C	31.1	5.3	1B/C	1BC
N,N-Dimethylisopropylamine	996-35-0	L	20.5	4.4	1B/C	17.8	3.3	1B/C	24.2	5.9	1B/C	1BC
Hydrochloric acid (14.4%)	7647-01-0	L	17.4	1.9	1B/C	21.6	1.0	1B/C	23.6	1.3	1B/C	1BC
Supplemental test												
Hydrochloric acid (10%)	*7647-01-0*	*L*	*57.0*	*6.8*	*1B/C*	*60.1*	*5.4*	*1B/C*	*59.4*	*9.8*	*1B/C*	*1BC*
Hydrochloric acid (18%)	*7647-01-0*	*L*	*7.4*	*1.1*	*1A*	*7.2*	*1.1*	*1A*	*5.6*	*1.1*	*1B/C*	*1A*
n-Heptylamine	111-68-2	L	8.7	2.3	1A	4.7	3.0	1A	12.8	1.6	1A	1A
Octanoic acid (caprylic acid)	124-07-2	L	10.7	5.4	1A	13.7	6.8	1A	13.1	7.0	1A	1A
Carvacrol	499-75-2	L	61.8	10.0	1B/C	35.2	7.4	1B/C	37.8	4.7	1B/C	1BC
2-Tert-Butylphenol	88-18-6	L	17.9	10.4	1B/C	25.0	2.9	1B/C	14.0	15.1	1A	1A(1T),1B/C(2T)
Methacrolein	78-85-3	L	33.9	3.8	1B/C	40.7	9.8	1B/C	64.7	10.3	1B/C	1B/C

Chemical	CAS number	State	Run 1			Run 2			Run 3			Final classification
			Viability (%)			Viability (%)			Viability (%)			
			3'	60'	Class	3'	60'	Class	3'	60'	Class	
Lactic acid	598–82–3	L	58.2	8.4	1B/C	52.1	1.0	1B/C	59.5	0.9	1B/C	1B/C
Sodium bisulphate monohydrate	10034–88–5	S	93.0	2.3	1B/C	65.2	0.6	1B/C	59.6	0.4	1B/C	1B/C
Glyoxylic acid monohydrate	563–96–2	S	77.6	1.9	1B/C	71.5	0.5	1B/C	62.9	0.4	1B/C	1B/C
Sodium bisulphate	7681–38–1	S	68.2	4.0	1B/C	30.8	2.4	1B/C	56.0	3.1	1B/C	1B/C
Cyclohexylamine	108–91–8	L	1.7	1.4	1A	1.2	2.1	1A	5.5	1.3	1A	1A
2–Methylbutyric acid	600–07–7	L	28.8	3.8	1B/C	20.4	1.6	1B/C	33.9	1.9	1B/C	1B/C
3–Methoxypropylamine	5332–73–0	L	21.1	3.3	1B/C	4.2	3.0	1A	2.0	1.4	1A	1A(2T),1B/C(1T)
Allyl bromide	106–95–6	L	49.9	4.9	1B/C	72.8	5.4	1B/C	76.1	11.1	1B/C	1B/C
1–(2–Aminoethyl)piperazine	140–31–8	L	37.5	6.8	1B/C	45.6	3.2	1B/C	42.1	9.0	1B/C	1B/C
Iron(III) chloride	7705–08–0	S	20.2	3.1	1B/C	53.2	4.8	1B/C	56.0	2.1	1B/C	1B/C
Phosphoric acid	7664–38–2	L	20.1	1.2	1B/C	16.4	0.9	1B/C	17.8	2.0	1B/C	1B/C
Propionic acid	79–09–4	L	8.1	1.1	1A	3.8	1.2	1A	2.0	1.1	1A	1A
Butyric acid	107–92–6	L	3.7	2.0	1A	9.1	1.2	1A	3.1	0.8	1A	1A
Boron trifluoride–acetic acid complex	373–61–5	L	1.4	0.3	1A	2.2	0.1	1A	2.1	0.2	1A	1A
Ethanolamine	141–43–5	V	20.8	3.2	1B/C	25.0	9.7	1B/C	26.9	6.9	1B/C	1B/C
Hydrobromic acid (48%)	10035–10–6	L	2.0	0.6	1A	2.1	0.4	1A	0.7	0.4	1A	1A
HCl + sulphuric acid + citric acid (5, 5, 5 wt%)		L	50.6	0.8	1B/C	32.7	0.7	1B/C	39.2	0.8	1B/C	1B/C
UN GHS Sub-category 1A based on in vivo results												
1,2–Diaminopropane	78–90–0	L	6.3	1.1	1A	12.6	13.0	1A	9.0	16.2	1A	1A
Phosphorus tribromide	7789–60–8	L	2.1	0.0	1A	2.2	0.3	1A	1.4	0.1	1A	1A
Boron trifluoride dehydrate	13319–75–0	L	0.7	28.6	1A	8.3	3.1	1A	2.2	1.0	1A	1A
Acrylic acid	79–10–7	L	1.9	0.5	1A	4.8	1.1	1A	14.5	0.9	1A	1A
Formic acid	64–18–6	L	1.0	0.2	1A	0.4	0.2	1A	0.4	0.6	1A	1A
Dichloroacetyl chloride	79–36–7	L	0.5	0.7	1A	0.2	1.5	1A	1.1	0.8	1A	1A
Silver nitrate	7761–88–8	S	0.5	0.6	1A	0.2	1.6	1A	0.8	0.2	1A	1A
Phenol	108–95–2	S	27.9	22.1	1B/C	13.8	11.4	1A	16.9	11.2	1B/C	1A(1T),1B/C(2T)
Acetic acid	64–19–7	L	1.7	1.3	1A	1.7	1.3	1A	2.8	1.8	1A	1A
Bromoacetic acid	79–08–3	S	2.9	1.1	1A	0.5	0.1	1A	0.6	0.4	1A	1A
N,N–dimethy–ldipropylenetriamine	10563–29–8	L	26.1	1.8	1B/C	40.4	1.2	1B/C	18.6	1.4	1B/C	1B/C
Sulphuric acid (98%)	7664–93–9	L	1.1	0.6	1A	2.1	1.2	1A	1.4	0.6	1A	1A

Abbrevitations: L = liquid; S = solid; V = viscous; 1T = one test; 2T = two tests

Underlined chemicals: reference chemicals listed in the Performance Standard (2)

ANNEX 2: MEAN CELL VIABILITY AND WITHIN-LABORATORY REPRODUCIBILITY (WLR)

No.	UN GHS *in vivo* Cat.	Lab A										Lab B										Lab C									
		Set 1			Set 2			Set 3			WLR	Set 1			Set 2			Set 3			WLR	Set 1			Set 2			Set 3			WLR
		3 min.	60 min.	J	3 min.	60 min.	J	3 min.	60 min.	J		3 min.	60 min.	J	3 min.	60 min.	J	3 min.	60 min.	J		3 min.	60 min.	J	3 min.	60 min.	J	3 min.	60 min.	J	
1	NC	104.5 (5.8)	118.1 (0.1)	NC	111.9 (1.1)	124.1 (2.3)	NC	96.1 (8.5)	104.6 (6.5)	NC	C	107.3 (0.5)	122.6 (5.9)	NC	124.9 (0.0)	103.5 (3.1)	NC	112.8 (1.6)	124.3 (1.0)	NC	C	94.8 (8.6)	130.1 (8.8)	NC	98.7 (3.5)	119.2 (14.1)	NC	101.6 (1.1)	119 (9.7)	NC	C
2	NC	96 (14.1)	77.1 (1.9)	NC	91.8 (1.9)	66.4 (3.2)	NC	93.6 (2.2)	72.2 (5.4)	NC	C	86.5 (4.0)	72.1 (4.6)	NC	114.2 (13.2)	81.3 (25.5)	NC	94.6 (2.3)	83.5 (10.6)	NC	C	103.2 (0.6)	100.1 (10.8)	NC	94.1 (3.7)	84.1 (0.1)	NC	82.4 (9.1)	98.8 (0.9)	NC	C
3	NC	100.2 (7.3)	112.3 6.8)	NC	106.2 (4.0)	104.4 (8.8)	NC	98.7 (7.2)	96.9 (1.8)	NC	C	98.1 (8.2)	101.0 (10.1)	NC	126.4 (1.0)	107.5 (7.3)	NC	102.2 (9.7)	129.9 (9.7)	NC	C	121.7 (17.2)	126.6 (26.7)	NC	125.6 (9.4)	109.2 (19.5)	NC	92.5 (3.8)	112.5 (18.0)	NC	C
4	NC	98.8 (16.4)	114.8 (0.6)	NC	95.5 (10.0)	107 (2.1)	NC	100.9 (0.7)	110.8 (0.9)	NC	C	100.5 (2.5)	105.8 (6.2)	NC	106.6 (2.0)	97.4 (8.0)	NC	100.6 (0.5)	106.7 (10.5)	NC	C	99 (6.4)	116.6 (21.1)	NC	95.2 (7.5)	123.7 (10.3)	NC	98.8 (1.1)	107.6 (0.9)	NC	C
5	NC	87.9 (10.0)	111.9 (11.7)	NC	94.7 (0.2)	107 (1.6)	NC	94.7 (3.4)	104.4 (4.1)	NC	C	96.3 (1.5)	94 (2.0)	NC	95.4 (2.0)	104.6 (1.5)	NC	100.1 (0.0)	109.3 (3.6)	NC	C	105.1 (1.9)	106.8 (7.5)	NC	108.9 (0.9)	120 (11.8)	NC	106.3 (3.6)	121.4 (3.5)	NC	C
6	NC	71.2 (8.4)	20 (2.8)	NC	65.2 (8.3)	20.7 (2.5)	NC	60.5 (2.8)	18.5 (0.1)	NC	C	78.8 (1.5)	19.6 (6.1)	NC	84.8 (23.4)	20.6 (4.0)	NC	65.3 (13.8)	24.1 (3.2)	NC	C	78.9 (0.9)	23.2 (0.6)	NC	75.5 (5.9)	20 (7.8)	NC	73.7 (1.4)	23.2 (1.3)	NC	C
7	NC	99.8 (5.4)	105.9 (1.3)	NC	100.4 (0.3)	96.3 (7.1)	NC	96.4 (2.3)	100.7 (0.7)	NC	C	97.4 (6.2)	99.1 (2.2)	NC	107.1 (1.0)	94.6 (9.1)	NC	94 (3.6)	100.5 (0.6)	NC	C	96.4 (0.4)	119.1 (9.6)	NC	98.3 (1.5)	101.2 (16.4)	NC	99.6 (1.6)	109.2 (11.6)	NC	C
8	NC	116 (13.6)	115.9 (9.5)	NC	94.5 (0.8)	98.3 (8.4)	NC	94.4 (0.8)	96.6 (0.7)	NC	C	102.9 (4.3)	94.2 (16.5)	NC	112.2 (7.1)	100.7 (13.2)	NC	94.8 (1.5)	104.3 (9.9)	NC	C	115.8 (5.8)	119.2 (5.4)	NC	95.9 (8.9)	100.6 (1.8)	NC	94.7 (13.2)	112.4 (2.3)	NC	C
9	NC	4.8 (2.5)	3.9 (6.5)	1A	6.8 (0.1)	7.6 (0.4)	1A	3.7 (0.0)	5.3 (2.7)	1A	C	5.7 (0.8)	12.7 (3.5)	1A	7.6 (1.0)	10.9 (0.4)	1A	6.3 (2.3)	2.2 (3.3)	1A	C	30 (27.8)	8.9 (3.9)	1B/C	58 (8.8)	0.3 (0.6)	1B/C	54.3 (1.0)	5.4 (6.6)	1B/C	C
10	NC	63.3 (7.2)	13.3 (3.9)	1B/C	71.4 (9.7)	15.6 (0.8)	NC	59.5 (11.8)	11.3 (0.5)	1B/C	N	86 (6.8)	18.2 (4.1)	NC	60.9 (2.0)	18.1 (4.2)	NC	85.4 (20.6)	17.4 (2.9)	NC	C	104.4 (1.5)	15.5 (0.3)	NC	111.6 (1.5)	15.5 (0.1)	NC	98.1 (4.3)	14.7 (1.2)	1B/C	N
11	1B/C*1	16.7 (0.6)	0.2 (0.0)	1B/C	15.4 (12.2)	0.6 (0.2)	1B/C	13.2 (4.4)	0.4 (0.1)	1A	N	12.2 (1.9)	2.5 (2.4)	1A	15.2 (8.1)	2 (1.9)	1B/C	65.6 (5.6)	2 (2.9)	1B/C	N	41.6 (22.4)	0.5 (0.0)	1B/C	63.4 (3.2)	0.7 (0.3)	1B/C	63.2 (0.3)	1.5 (2.3)	1B/C	C

No.	UN GHS *in vivo* Cat.	Lab A										Lab B										Lab C									
		Set 1			Set 2			Set 3			WLR	Set 1			Set 2			Set 3			WLR	Set 1			Set 2			Set 3			WLR
		3 min.	60 min.	J	3 min.	60 min.	J	3 min.	60 min.	J		3 min.	60 min.	J	3 min.	60 min.	J	3 min.	60 min.	J		3 min.	60 min.	J	3 min.	60 min.	J	3 min.	60 min.	J	
12	1B/C	5.2 (1.0)	0.5 (0.4)	1A	3.5 (0.3)	4 (1.6)	1A	2.6 (0.1)	3.5 (0.4)	1A	C	5.8 (2.9)	1.4 (2.7)	1A	6.1 (2.0)	4.4 (4.4)	1A	7.8 (3.8)	2.6 (2.8)	1A	C	~~40.1 (46.7)~~	0.5 (0.3)	Invalid test run	8.7 (8.5)	0.9 (0.6)	1A	62.9 (24.9)	0.4 (0.3)	1B/C	N
																						Re-test 01									
																						0 (0.0)	0 (0.0)	1A							
13	1B/C	50.3 (18.9)	2.4 (0.1)	1B/C	43.8 (9.4)	0.9 (0.1)	1B/C	47.8 (1.5)	1.6 (0.6)	1B/C	C	66.1 (5.6)	3.3 (2.6)	1B/C	69 (2.0)	3.4 (2.2)	1B/C	70 (2.8)	1.5 (1.9)	1B/C	C	78.3 (3.5)	6.1 (0.3)	1B/C	79.1 (13.1)	4.2 (5.4)	1B/C	72 (2.7)	3.9 (2.3)	1B/C	C
14	1B/C	19.5 (1.6)	6.2 (2.7)	1B/C	20.2 (0.0)	2.6 (0.8)	1B/C	23.5 (5.2)	2 (1.9)	1B/C	C	21.7 (14.1)	9.6 (2.2)	1B/C	50.8 (6.1)	4.2 (3.8)	1B/C	38.7 (5.5)	4.5 (2.0)	1B/C	C	67.4 (0.9)	8.9 (11.0)	1B/C	57.4 (6.9)	11.2 (0.9)	1B/C	54.9 (2.6)	4.2 (0.4)	1B/C	C
15	1B/C	23.7 (9.2)	4.7 (1.1)	1B/C	76.2 (1.9)	4.7 (0.4)	1B/C	32.5 (7.0)	4.3 (1.0)	1B/C	C	34.2 (1.4)	5.5 (3.6)	1B/C	21.3 (0.0)	1.1 (2.3)	1B/C	23.3 (4.8)	4.4 (2.0)	1B/C	C	43.6 (6.9)	11.5 (1.3)	1B/C	28.6 (13.3)	0.7 (0.1)	1B/C	22.3 (7.9)	10.3 (0.8)	1B/C	C
16	1B/C	3.9 (0.2)	0.9 (0.6)	1A	2.4 (0.2)	0.7 (0.1)	1A	2.1 (0.8)	0.7 (0.5)	1A	C	4 (2.8)	2.8 (3.9)	1A	2.5 (3.0)	1.5 (1.9)	1A	5.4 (2.1)	2.2 (2.6)	1A	C	3.3 (3.2)	3.4 (2.9)	1A	6 (6.1)	1.8 (3.0)	1A	7.3 (3.1)	1.7 (0.6)	1A	C
17	1B/C	0.7 (0.1)	0.2 (0.1)	1A	0.5 (0.2)	0.7 (0.9)	1A	0.5 (0.0)	0.4 (0.1)	1A	C	2.4 (2.1)	2.9 (1.6)	1A	3.6 (1.0)	1.3 (2.2)	1A	2 (1.8)	1.5 (3.1)	1A	C	0.3 (0.5)	0.1 (0.3)	1A	1 (0.5)	0.5 (0.0)	1A	1.1 (0.1)	0.3 (0.1)	1A	C
18	1B/C	1.1 (0.5)	1 (0.1)	1A	1.5 (0.1)	1.4 (0.1)	1A	1.5 (0.0)	1.9 (0.9)	1A	C	2.2 1.2)	3 (1.6)	1A	2.5 (1.0)	2 (2.1)	1A	2.4 (2.0)	2.8 (3.1)	1A	C	2.6 (0.9)	0.7 (0.2)	1A	1.4 (0.2)	1.1 (0.9)	1A	2 (0.2)	1.7 (0.4)	1A	C
19	1B/C	6 (1.4)	7.2 (5.4)	1A	20.5 (0.5)	14 (0.5)	1B/C	12.8 (0.0)	0 (0.0)	1A	N	16.4 (0.9)	1.7 (3.4)	1B/C	33.5 (10.2)	17.1 (3.4)	1B/C	22.7 (6.1)	13.6 (2.7)	1B/C	C	6.8 (13.7)	0 (0.0)	1A	8 (1.8)	0 (0.0)	1A	10.8 (0.3)	16.6 (7.6)	1A	C
20	1B/C	0.6 (0.2)	0.4 (0.0)	1A	0.9 (0.1)	0.6 (0.2)	1A	0.5 (0.1)	0.3 (0.0)	1A	C	2.3 (1.5)	7.3 (0.1)	1A	6.6 (1.0)	3.5 (2.2)	1A	3.2 (0.0)	2 (2.9)	1A	C	1.4 (0.3)	3.6 (2.9)	1A	1.2 (0.4)	3.5 (0.8)	1A	1.3 (0.3)	0 (0.0)	1A	C
21	1A*3	0.4 (0.1)	3.1 (0.1)	1A	0.6 (0.0)	0.7 (0.0)	1A	0.7 (0.2)	1.1 (0.2)	1A	C	3 (2.0)	3.9 (2.2)	1A	2 (2.0)	1.8 (2.3)	1A	2.2 (1.5)	2.2 (2.1)	1A	C	0.6 (0.0)	1.6 (0.0)	1A	0.6 (0.2)	2 (0.4)	1A	0.6 (0.4)	0.6 (0.0)	1A	C
22	1A	0.8 (0.5)	0.5 (0.6)	1A	1 (0.6)	0.6 (0.0)	1A	0.7 (0.1)	0.3 (0.2)	1A	C	2 (1.9)	2.2 (1.1)	1A	~~3.1 (2.0)~~	~~49.0 (97.3)~~	Invalid test run	1 (0.9)	1.3 (2.6)	1A	C	1.4 (1.0)	0.5 (0.7)	1A	0.3 (0.1)	1 (1.0)	1A	0.6 (0.2)	0.3 (0.1)	1A	C
															Re-test 01																
															1.0 (1.0)	1.0 (1.0)	1A														

No.	UN GHS *in vivo* Cat.	Lab A										Lab B										Lab C									
		Set 1			Set 2			Set 3			WLR	Set 1			Set 2			Set 3			WLR	Set 1			Set 2			Set 3			WLR
		3 min.	60 min.	J	3 min.	60 min.	J	3 min.	60 min.	J		3 min.	60 min.	J	3 min.	60 min.	J	3 min.	60 min.	J		3 min.	60 min.	J	3 min.	60 min.	J	3 min.	60 min.	J	
23	1A	7.8 (2.5)	1 (0.5)	1A	8.2 (0.9)	1.7 (0.0)	1A	6.8 (0.1)	1.1 (0.9)	1A	C	9 (3.1)	1.6 (3.2)	1A	12.2 (2.0)	2.8 (3.8)	1A	5.3 (6.7)	1.4 (2.3)	1A	C	7.3 (0.3)	5.5 (0.2)	1A	8.6 (0.5)	1.3 (0.3)	1A	4.9 (0.9)	2.8 (1.5)	1A	C
24	1A	14.4 (2.8)	7.6 (1.3)	1A	13.9 (2.2)	7.2 (0.1)	1A	11.4 (1.7)	4.3 (2.4)	1A	C	21.3 (6.8)	9.7 (4.0)	1B/C	18.8 (5.1)	10.1 (1.7)	1B/C	15.4 (0.7)	10.5 (3.4)	1B/C	C	15.5 (1.2)	10.5 (0.7)	1B/C	13.1 (1.2)	7.3 (0.5)	1A	10 (0.8)	8.1 (1.5)	1A	N
25	1A	0.4 (0.2)	0.1 (0.0)	1A	3.1 (0.1)	0.1 (0.1)	1A	2.9 (0.7)	0.3 (0.3)	1A	C	1.7 (3.2)	1.3 (2.6)	1A	4.1 (2.0)	0.8 (1.6)	1A	2.6 (1.2)	1.1 (2.3)	1A	C	0.2 (0.3)	0 (0.0)	1A	0.9 (0.7)	0.6 (0.1)	1A	1.3 (1.0)	0.4 (0.3)	1A	C
26	1A	0 (0.0)	0.1 (0.2)	1A	0 (0.0)	0 (0.0)	1A	0 (0.0)	0 (0.0)	1A	C	1.4 (1.7)	2.4 (4.6)	1A	2.5 (1.0)	2.9 (5.6)	1A	1.4 (2.3)	1.5 (3.0)	1A	C	1.7 (2.4)	0.3 (0.0)	1A	0.1 (0.3)	1.3 (0.6)	1A	1.4 (1.7)	0.3 (0.5)	1A	C
27	1A	0.3 (0.1)	0.1 (0.0)	1A	0.3 (0.3)	0.1 (0.2)	1A	0.2 (0.1)	0.5 (0.9)	1A	C	0.8 (1.5)	1.5 (0.2)	1A	1 (2.0)	1 (2.0)	1A	1.8 (0.4)	1.2 (2.5)	1A	C	0 (0.0)	0.1 (0.2)	1A	0.1 (0.1)	0 (0.0)	1A	0 (0.0)	0.1 (0.1)	1A	C
28	1A	0.3 (0.1)	0.2 (0.0)	1A	0.3 (0.4)	0.4 (0.1)	1A	0.7 (1.1)	0.4 (0.0)	1A	C	1.1 (2.1)	1.2 (2.5)	1A	1.5 (1.0)	0.9 (1.9)	1A	0.7 (0.7)	0.6 (1.3)	1A	C	0.1 (0.3)	0 (0.0)	1A	0.4 (0.1)	0.3 (0.3)	1A	0.5 (0.4)	0 (0.1)	1A	C
29	1A	0.1 (0.0)	0.1 (0.1)	1A	0.4 (0.1)	0.1 (0.0)	1A	0.2 (0.1)	0.5 (0.3)	1A	C	5.5 (0.7)	1.2 (2.5)	1A	3.1 (2.0)	0.5 (1.0)	1A	1.7 (1.6)	1.3 (2.7)	1A	C	2.6 (3.7)	0.3 (0.7)	1A	1.1 (2.1)	0.6 (0.1)	1A	0.8 (1.2)	0.3 (0.7)	1A	C
30	1A	37.5 (9.9)	2.2 (0.2)	1B/C	44.5 (16.1)	1.6 (0.1)	1B/C	37.2 (6.0)	1.6 (0.8)	1B/C	C	25.7 (4.0)	4.6 (1.4)	1B/C	31.5 (8.1)	3.1 (2.2)	1B/C	62.6 (3.0)	3.5 (1.7)	1B/C	C	55 (5.8)	4.9 (1.1)	1B/C	72.5 (5.0)	6.2 (1.0)	1B/C	65.8 (9.0)	5.3 (0.6)	1B/C	C

Upper row: viability in %, Lower row (in brackets): difference in viability in %.
Orange cells indicate an invalid test run.
Abbreviations: J = Judge; NC = Not corrosive; 1B/C: Sub-categories 1B-and-1C; 1A = Sub-categories 1B-and-1C;
WLR: Within-laboratory reproducibility, C: Concordance, N: Non-concordance